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Novel approaches to the development and assessment of an ovine model of polycystic ovary syndrome

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Declaration

I hereby declare that the work presented in this thesis was carried out by the author, and where this is not the case, this is referred to in the text. This thesis is submitted for the fulfilment of the degree of Doctor of Philosophy, and not for any other degree or qualification.

Kirsten Hogg

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Abstract

Polycystic ovary syndrome (PCOS) is a common reproductive, endocrine and metabolic disorder present in women of reproductive age. Despite the widespread prevalence and heritability of PCOS, the heterogeneous and polygenic traits have made the successful identification of candidate genes difficult. Animal models have been developed on the premise that early exposure to sex steroids can programme epigenetic changes that predispose the fetus to the adult features of PCOS. Past research has modelled ovarian dysfunction, endocrine abnormalities and metabolic perturbances in rodent, non-human primate and sheep PCOS models, through the enhanced neonatal or prenatal exposure to the male sex hormone, testosterone.

The modelling of PCOS in a large domestic species such as the sheep is advantageous due to similar biological reproductive function as the human. In this regard the sheep has been extensively used to model PCOS by the treatment of pregnant ewes from early to mid-gestation with androgens such as testosterone propionate (TP). These experiments have demonstrated the fetal programming effects of androgens on offspring that go on to develop PCOS-like characteristics in adulthood. One of the caveats of assessing steroid effects in this way is the effect of the placenta in mediating the transfer of these hormones. TP is an aromatisable androgen and thus some of its effects in the fetus may be attributable to placental by-products such as estrogens.

This thesis describes the development and assessment of a novel model of prenatal androgenisation. Two models were compared: the indirect maternal exposure to TP (the current model) and the direct fetal injection of TP. In directly treating the fetus this allowed control over the dose of TP administered and avoidance of secondary effects that androgens may exert in the mother that could be transferred to the fetus. For the maternal model, pregnant Scottish Greyface ewes were administered TP twice weekly from day (d)62-102 of a 147 day gestation. For the fetal model, fetuses were injected twice while the ewe was anaesthetised with graded doses of TP during the same period of treatment as the maternal model.

The effects of prenatal androgenisation were assessed in the female fetus shortly after treatment and also in young adult sheep. Fetal ovarian and adrenal steroidogenic gene

expression was monitored and found to be altered in response to elevated levels of sex steroids. At d90 the morphology of the developing ovary was not changed by prenatal androgens. In the adult a detailed ovarian and endocrine assessment was undertaken, by examination of ovarian morphology, hormone levels, ovulatory cycles, hypothalamic pituitary ovarian function and follicle steroidogenesis, during the first breeding season. In addition, the metabolic effects of prenatal androgens were monitored by measuring body fat, insulin and glucose homeostasis and liver function. Neither maternal nor fetal prenatal androgenisation during mid-gestation resulted in a perturbed hormonal milieu or polycystic ovaries in young adults. These treatments did however programme a clear ovarian phenotype demonstrated by the increased capacity of follicles to secrete androgens, independently of an abnormal endocrine environment and disordered folliculogenesis. Furthermore, animals that were exposed maternally to TP developed fatty liver and had increased insulin secretion in response to glucose load. A major outcome of this study was the finding that the fetally injected control animals were phenotypically different than the maternal control animals. In fact, some of the reproductive and metabolic features of maternal TP exposure were found in the fetal control group. This unexpected finding has raised the possibility that it is the fetal exposure to stress, that is secondary to elevated maternal androgens, rather than androgens *per se* that is responsible for at least some of the multitude of anomalies encountered in PCOS.

Presentations and publications relating to this thesis

Chapter 4: The effect of prenatal androgens on the developing ovary

Paper

Hogg K, McNeilly AS, Duncan WC (2011) Prenatal Androgen Exposure Leads to Alterations in Gene and Protein Expression in the Ovine Fetal Ovary. Endocrinology 152:2048-2059

Abstract

Hogg K, McNeilly AS, Duncan WC (2010) Indirect and Direct Effects of Increased Androgen Exposure In Utero on the Ovine Fetal Ovary. (Poster Presentation; SSR Annual Meeting, July 2010, Milwaukee, USA)

Chapter 5: The characterisation of Id expression in the adult ovary and their regulation by TGFβ factors

Paper

Hogg K, Etherington SL, Young JM, McNeilly AS, Duncan WC (2010) Inhibitor of Differentiation (Id) Genes Are Expressed in the Steroidogenic Cells of the Ovine Ovary and Are Differentially Regulated by Members of the Transforming Growth Factor-β Family. Endocrinology 151:1247-1256

Abstract

Hogg K, Etherington SL, Young JM, McNeilly AS, Duncan WC (2009) Localisation of inhibitor of differentiation (Id) proteins in the sheep ovary. (Poster Presentation; SRF Annual Meeting July 2009, Oxford, UK).

Chapter 7: The effect of prenatal androgen on ovarian function and folliculogenesis

Paper

Hogg K, Young JM, Oliver E, Sousa CJ, McNeilly AS, Duncan WC (2011) Enhanced thecal androgen production is prenatally programmed in an ovine model of polycystic ovary syndrome. Endocrinology. *In Review*

Abstract

Hogg K, Young JM, Sousa CJ, McNeilly AS, Duncan WC (2011) Enhanced thecal cell steroidogenesis is programmed in an ovine model of polycystic ovary syndrome (PCOS). (Oral presentation; SRF Annual Meeting, July 2011, Brighton, UK)

Chapter 9: The effect of prenatal androgens on metabolic parameters

Paper

Hogg K, Wood C, McNeilly AS, Duncan WC (2011) The *in utero* programming effect of increased maternal androgens and a direct fetal intervention on liver and metabolic function in adult sheep syndrome. PLoS One 6(9): e24877. doi:10.1371/journal.pone.0024877

Abstracts

Hogg K, McNeilly AS, Duncan WC (2011) Early incidence of fatty liver and metabolic disturbance in an ovine intrauterine model of increased androgen exposure. (Poster presentation; SSR Annual Meeting, August 2011, Portland, USA)

Hogg K, McNeilly AS, Duncan WC (2010) Fetal Programming: Direct and Indirect Fetal Androgenisation Differentially Regulate the Metabolic Phenotype in Adult Sheep (Oral Presentation; SRF Annual Meeting, July 2010, Nottingham, UK)

General

Invited Seminar

Hogg K (2010) The programming impact of sex steroids on fetal development: metabolic, endocrine and ovarian pathophysiology in adult offspring (Aug 2010, Child and Family Research Institute, University of British Columbia, Vancouver, Canada)

Abstract

K Hogg, AS McNeilly, WC Duncan (2009) The fetally androgenised sheep: a novel approach in investigation of polycystic ovary syndrome (PCOS). (Oral presentation, NOW Biannual Meeting, July 2009, Cambridge, UK)

Abbreviations

ACTH - adrenocorticotropic hormone

ACVR - activin receptor

AGD - ano-genital distance

ALP - alkaline phosphatase

ALT - alanine aminotransferase

AMH - anti-müllerian hormone

ANOVA - analysis of variance

APS - ammonium persulphate

AR - androgen receptor

AST - aspartate aminotransferase

B0 - zero binding

bHLH - basic helix loop helix

BLAST - Basic Local Alignment Search Tool

BMI - body mass index

BMP - bone morphogenetic protein

Bp - base pair

BSA - bovine serum albumin

cDNA - complementary deoxyribonucleic acid

cpm - counts per minute

Ct - cycle threshold

CV - co-efficient of variance

CYP - cytochrome

DAB - 3,3'-diaminobenzidine

DAPI - 4',6-diamidino-2-phenylindole

DES - diethylstilbestrol

DEX - dexamethasone

dH_2O - distilled water

DHEA(S) - dehydroepiandrosterone (sulphate)

DHT - dehydrotestosterone

DNA - deoxyribonucleic acid

dNTP - deoxynucleotide triphosphate

ds- double stranded

ELISA - enzyme-linked immunosorbent assay

ER - estrogen receptor

FFA - free fatty acids

FI - fetal injection

FSH(R) - follicle stimulating hormone (receptor)

GAMB - goat anti-mouse biotinylated

GAPDH - glyceraldehyde 3-phosphate dehydrogenase

GARB - goat anti-rabbit biotinylated

GDF - growth differentiation factor

GGT- gamma glutamyltransferase

GH - growth hormone

GnRH(R) - gonadotrophin releasing hormone (receptor)

GR - glucocorticoid receptor

GTT - glucose tolerance test

H&E - haematoxylin and eosin

HPA - hypothalamic pituitary adrenal

HPO - hypothalamic pituitary ovarian

HRP - horse radish peroxidase

HSD - hydroxysteroid dehydrogenase

Id - inhibitor of differentiation

IGF(R) - insulin growth factor (receptor)

IGFBP - insulin-like growth factor binding protein

IgG - immunoglobulin

INSR - insulin receptor

IUGR - intrauterine growth restriction

LH(R) - luteinising hormone (receptor)

MI - maternal injection

mRNA - messenger ribonucleic acid

Mw - molecular weight

NAFLD - non-alcoholic fatty liver disease

NCBI - National Center for Biotechnology Information

NF - nuclease free

NGS - normal goat serum

NIAMDD - National Institute of Arthritis, Metabolism and Digestive Diseases

NIDDK - National Institute of Diabetes and Digestive and Kidney Diseases

NIH - National Institutes of Health

NSB - non-specific binding

OVX - ovariectomised

PAGE - polyacrylamide gel electrophoresis

PBS(T) - phosphate buffered saline (tween)

PCO - polycystic ovaries

PCR - polymerase chain reaction

PCOS - polycystic ovary syndrome

PEPCK - phosphoenolpyruvate carboxykinase

PGBS - phosphate gelatin buffer saline

PGC - primordial germ cell

PND - post-natal day

POMC - pro-opiomelanocortin

qRT-PCR - quantitative real time - polymerase chain reaction

RAGB - rabbit anti-goat biotinylated

RIA - radioimmunoassay

RNA - ribonucleic acid

ROBO - roundabout

RT - reverse transcriptase

SDS - sodium dodecyl sulfate

SGA - small for gestational age

SHBG - sex hormone binding globulin

StAR - steroidogenic acute regulatory protein

SULF2A1 - sulfotransferase 2A1

T0 - total counts

TBS(T) - tris buffered saline (tween)

TEMED - tetramethylethylenediamine

TF - transcription factor

TGF - transforming growth factor

TMB - 3,3’,5,5’-tetramethylbenzidine

TP - testosterone propionate

UV - ultra violet

VEGF - vascular endothelial growth factor

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Chapter 1

Literature Review

The endocrine system encompasses a precise balance of circulating hormones that are crucial for homeostatic maintenance and the control of normal physiological functions, including reproduction and metabolism. A series of tightly controlled feedback loops link these systems together to form highly efficient processes that maintain homeostatic equilibrium. The hypothalamic pituitary ovarian (HPO) axis is an example of such a process. It is the bi-directional passage of hormonal signals along this axis that regulates the timing of puberty and the whole sequence of events that are required for initiation of regular menstrual cycles, implantation of the conceptus and the maintenance of pregnancy. A separate set of hormonal signals are released by the brain to stimulate another endocrine gland, the adrenal, forming the hypothalamic pituitary adrenal (HPA) axis. The adrenal gland is a major source of steroids, in addition to the gonads, secreting androgens, mineralocorticoids and glucocorticoids. It is particularly important in the regulation of physiological stress, initiating the 'fight or flight' response and maintaining kidney function. The metabolic system also plays a central role in the regulation of the HPO and HPA axes, which in turn, can finely tune the processes that are involved in lipid accumulation and fat distribution and contribute to maintaining normoglycaemia. A major role for insulin in the control and maintenance of a range of metabolic and non-metabolic tissues is realised with peripheral targets including muscle, fat, liver, skin, adrenals, bone and the ovary.

Dysregulation of this delicate balance of hormonal signals, which act in a complex manner to organise congruent tissue and body function, results in pathophysiologies associated with a multitude of endocrine, reproductive and metabolic disturbances. One such outcome is a common disorder in women termed polycystic ovary syndrome (PCOS) that can encompass all three of these disturbances. Dissecting out the origin(s) of PCOS, whether genetic or epigenetic, has thus far been challenging, particularly due to the heterogeneity of the disorder. This literature review will describe early mammalian reproductive development and adult reproductive function, detail the current known aetiology for PCOS and treatment strategies, and discuss how the ovary, adrenal and metabolic systems are intertwined to produce the features of PCOS in women. In parallel, the various animal models of PCOS, in which the female fetus is exposed to increased androgens *in utero*, and how these contribute to the current body of knowledge concerning PCOS, will be described.

1.1 Mammalian reproductive function

During fetal development the gonadal ridge and mesonephroi (primitive kidneys) form the urogenital ridge, from which the sexually indifferent gonads arise at around the fourth week of gestation in the human (1) and between 23-24 days in the sheep fetus (2). Primordial germ cells (PGCs) migrate to and enter the gonad around the time of gonad formation (3), proliferating so that a large reserve of germ cells populate the developing gonad. From a founder pool of approximately 100 PGCs (3), as many as 250,000 germ cells are predicted to be present in the human ovary by 10 weeks of development (4). Gonadal sexual differentiation occurs soon after the establishment of the germ cell population, at 7 weeks of gestation in humans (5) and between d32-35 in sheep (2). In the female, primordial germ cells, termed gonocytes, differentiate into oogonia and undergo further mitosis before entering meiosis which is subsequently arrested during prophase I (6). Oocytes are first detected in the human ovary at around 16 weeks of gestation (7) and while estimates of the total number at this stage vary (8), peak oocyte numbers occur in the mid-gestation ovary. However, a great number will degenerate through atresia before follicle formation. Primordial follicles consist of an oocyte surrounded by a layer of flattened or squamous granulosa cells and are assembled during fetal development in humans and domestic animals (9, 10). The first primordial follicles form at around d75 in the fetal sheep ovary (11). It is at this time that the majority of oocytes undergo programmed cell death, known as atresia. During the third trimester of pregnancy follicles can grow as far as the antral stage (Section 1.1.1) in the human and sheep fetal ovary (12). An illustration of the timeline for gonadal formation, sexual differentiation and the timing of primordial follicle assembly in the sheep is provided in Figure 1.1. At birth the mammalian ovary is thought to contain a finite number of primordial follicles (13, 14) reported to be in the region of 1 million follicles in the human (7, 15) and approximately 70-90,000 in the sheep (16), although some reports attempt to contradict this dogma (17). Primordial follicles are quiescent for much of an individual's life and will either have one of two fates, being activated to progress through transitionary follicles stages until follicular dominance is reached and ovulation of a developmentally competent oocyte occurs, or more commonly, follicles will undergo atresia, which will occur in the majority of the follicle population (10, 18). From an estimated 0.5 million primordial follicles present at the beginning of menarche in women, only about 400 are ovulated during an average un-interrupted ovulatory lifespan (7).

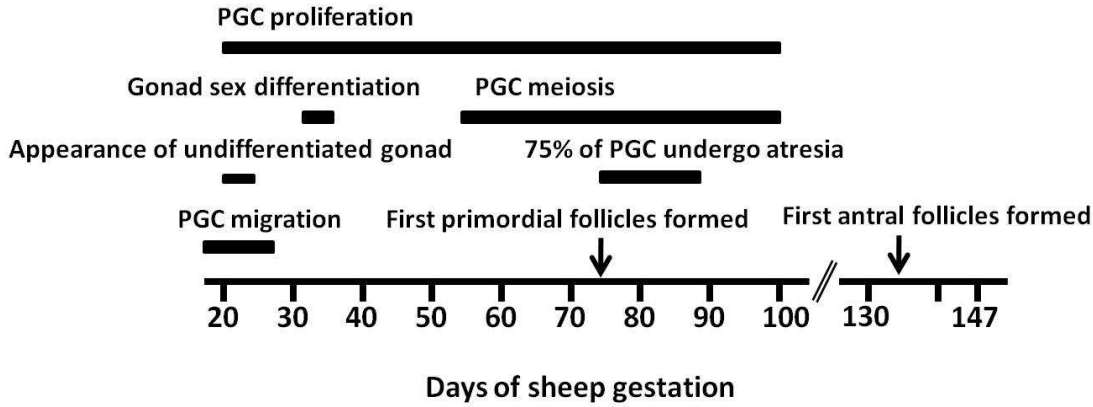

Figure 1.1 Ovine gonadal development. The developmental timeline for gonad formation, PGC migration, proliferation and meiosis, and the first appearance of activated follicles in the fetal sheep.

Adapted from Juengel *et al.* 2002 (2)

1.1.1 Follicle development

In the ovary, primordial follicles are activated, and undergo transition into progressive stages of follicle development, which can take weeks to months to complete (7, 19). The oocyte enlarges over this time as ribonucleic acid (RNA) is accumulated and protein is synthesised and it gains the competence for fertilisation (20). The human oocyte will increase in size from ~20µm to 60-120µm but meiosis is not resumed (21). The oocyte becomes surrounded and is nursed by somatic ovarian cells, including granulosa cells and thecal cells, which undergo mitosis so that the whole follicle diameter becomes much larger over development while the overall increase in oocyte volume is initially much less (22). In the sheep, oocyte and follicle diameter get larger concomitantly during early follicular growth (23). From the resting primordial stage, follicles enter the primary (pre-antral) stage which is distinguished by the 'plumping' up of the surrounding granulosa cells into a single layer of cuboidal cells, which begin to proliferate (13) (Fig. 1.2). The oocyte secretes glycoproteins forming a surrounding layer called the zona pellucida that separates the oocyte from the granulosa cell layer (24). The chief function of the zona pellucida is related to the binding of sperm and induction of the acrosomal reaction, facilitating entry into the oocyte (25, 26). The zona pellucida contains numerous gap junctions to allow for the bi-directional flow of paracrine factors secreted by the oocyte and granulosa cells (27).

In the secondary (antral) stage follicle, granulosa cells continue to proliferate forming multiple layers. The formation of a basement membrane around the outer edge of the granulosa layer separates these cells from the thecal layer (Fig. 1.2). Theca cells are recruited from the surrounding stroma and interstitium during the later secondary stage of follicular development in domestic species such as the cow and sheep (23, 28). The thecal layer is composed of the theca interna, containing many blood vessels, and the theca externa, the outer capsule (20). In early secondary stage follicles there is formation of fluid filled antral spaces within the granulosa layer and these follicles are termed small antral follicles (Fig. 1.2). In large antral follicles a large antral cavity filled with follicular fluid coalesces, separating the cumulus granulosa cells directly adjacent to the oocyte from mural granulosa cells adjacent to the basement membrane (21). These follicles continue to grow until a dominant follicle is selected for ovulation. The pre-ovulatory (tertiary) follicle (Fig. 1.2) continues to grow at the expense of the subordinate antral follicles which undergo atresia. Approximate follicle diameter sizes for each developmental stage in the human and sheep ovary are detailed in Table 1.1.

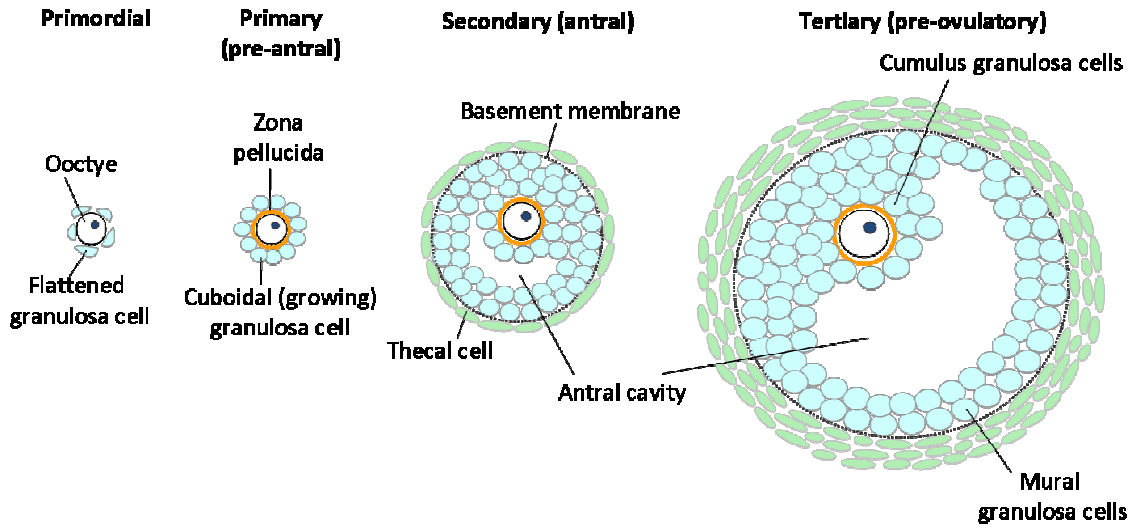

Figure 1.2 Ovarian follicle development. The growth trajectory of ovarian follicles from the resting primordial stage to the pre-ovulatory stage. The somatic cells, the granulosa and thecal cells, house the oocyte in a follicular unit and support oocyte maturation and acquisition of developmental competence. The complete process of follicle development (so that the oocyte is ready to ovulate) takes at least 85 days in the human (24) and ~5 months in the sheep (29).

1.1.2 The ovulatory cycle

In pre-pubertal girls, the HPO axis is suppressed, the ovary is quiescent and gonadotrophin and sex hormones are therefore present at low levels. Puberty is triggered by the activation of hypothalamic gonadotrophin releasing hormone (GnRH) secretion and is associated with a growth spurt, and in females, the beginning of breast development (21). Menarche follows 1-2 years later and is directly correlated with body mass, particularly body fat composition. In general a threshold weight of 47 kg has been postulated to be necessary to initiate adult reproductive function (21). The human menstrual cycle spans an approximate 28 day period, and is split into two distinct phases, the follicular phase and the luteal phase, which are separated by ovulation on day 14 (Fig. 1.3).

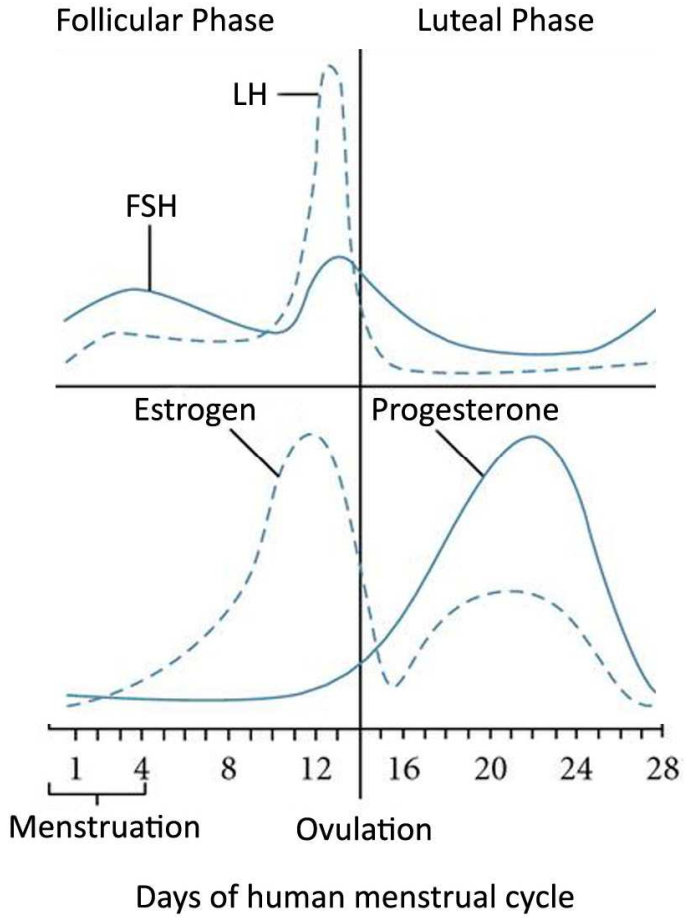

Figure 1.3 The hormonal profile over the human menstrual cycle.

Adapted from Shechter and Boivin 2009 (30)

The HPO axis is the physiological circuitry that is responsible for initiation and maintenance of ovulation and menstruation in females. Signalling is initiated in the hypothalamus, which is situated below the thalamus at the base of the brain, and contains numerous nuclei that control physiological homeostasis such as thirst and hunger as well as blood pressure and heart rate (31). A major endocrine role of the hypothalamus is the release of a variety of neurohormones which travel via the median eminence to the pituitary gland, located below the hypothalamus in the fossa of the sphenoid bone (21). The pituitary is split into two distinct glands: the anterior and posterior lobe. The posterior pituitary is the site of vasopressin and oxytocin synthesis. The anterior pituitary contains a number of highly specialised cell types including the gonadotrophs, thyrotrophs, lactotrophs, somatotrophs and corticotrophs that are each responsible for producing and secreting specific hormones that have vital roles in maintaining homeostasis. The thyrotrophs, lactotrophs, somatotrophs and corticotrophs synthesise thyroid stimulating hormone, prolactin, growth hormone (GH) and pro-opiomelanocortin (POMC) the precursor of adrenocorticotrophin hormone (ACTH), respectively, which have non-gonadal targets and each are stimulated by their own specific releasing hormone produced by the hypothalamus (32). The gonadotrophs are basophilic cells which synthesise, store and secrete the gonadotrophins, luteinising hormone (LH) and follicle stimulating hormone (FSH) (33), which are released from gonadotrophs upon pulsatile stimulation by the hypothalamic decapeptide GnRH that binds to GnRH receptors (GnRHR) situated on the gonadotroph (32). The expression of GnRHR can be regulated by GnRH, and is up-regulated by pulsatile GnRH neuronal firing, and conversely de-sensitised by sustained GnRH stimulation (34, 35).

LH and FSH are heterodimeric glycoproteins that are made up of a non-covalently linked common α subunit and a specific β subunit (36), of which all three types of subunit expression are stimulated by GnRH (34). While LH and FSH are synthesised and stored by the same cell type, and both stimulated through GnRH signalling, their biological properties are quite different (37). LH has a very short half-life of just 20 min and protein turn over is high and short-lived so that the pulsatile pattern of this hormone is maintained. Every time there is a burst of GnRH activity, there is a subsequent release of LH, followed by a decline before the next round of neuronal GnRH firing is initiated. On the other hand, FSH has a longer half life than LH, and secretion does not follow a pulsatile pattern (38). LH receptors (LHR) and FSH receptors (FSHR) are expressed by thecal cells and granulosa cells, respectively, by the pre-antral stage of follicle development (39, 40). LHR are also up-

regulated in the granulosa cells of large pre-ovulatory follicles, as they mature as part of follicular dominance and preparation for ovulation (39, 40). The gonadotrophins act in an endocrine manner on the ovary, to promote folliculogenesis, ovulation and subsequent luteal function.

Primordial follicle initiation and early follicle growth are not completely dependant on gonadotrophins, since a hypopituitary state does not prevent these processes occurring (41). Instead gonadotrophins may act in combination with other factors to regulate growth (42-44). However, the presence of circulating FSH is essential for the growth of large antral follicles. FSH stimulates the expression of the steroidogenic enzyme, aromatase, and promotes the induction of LHR, in granulosa cells of antral follicles (45). During the latter part of the luteal phase and early follicular phase FSH levels increase (Fig. 1.3) to promote the growth of healthy antral follicles (46), which are approximately 2-5mm in diameter in women (47). At the same time LH is secreted from the pituitary in a pulsatile manner as a direct consequence of pulsatile firing of hypothalamic GnRH neurons. The frequency of pulsatile LH secretion increases to approximately one per hour during the follicular phase, and is required for maintenance of antral follicle development (45, 48).

The presence of LH, in combination with FSH, is responsible for ovarian steroid production by growing follicles through the two-cell, two-gonadotrophin mechanism (14, 40, 49) (Fig. 1.4). Thecal cells produce androgens through a series of steroidogenic steps upon binding of LH to the LHR. Androstenedione is synthesised and diffuses to the granulosa cell compartment where it is converted to estrone and estradiol, by aromatase, under the influence of FSH binding to the FSHR (14). In addition to acting as a substrate for estradiol synthesis, thecal androgens, including testosterone and androstenedione, may also augment granulosa cell aromatase activity through binding to androgen receptors (AR) present on granulosa cells (50, 51).

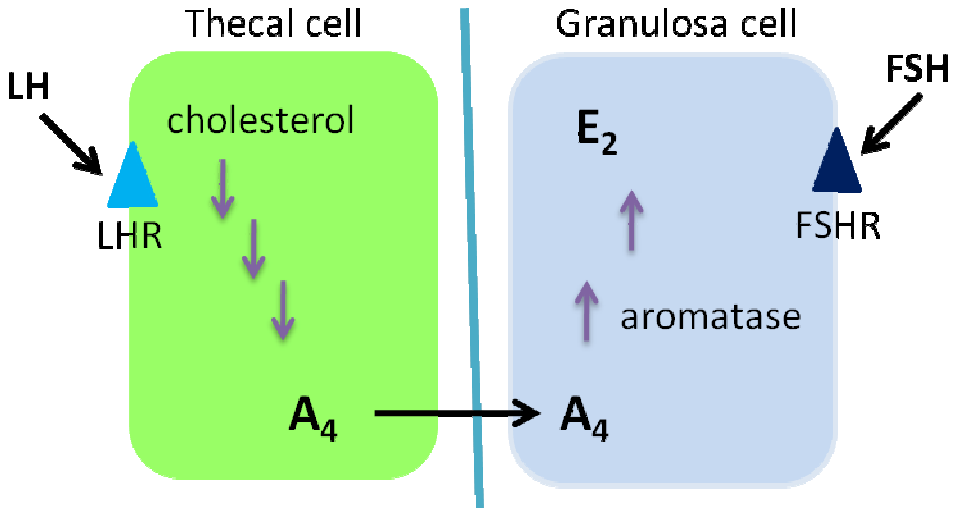

Figure 1.4 The two cell, two gonadotrophin mechanism. The cartoon illustrates the synthesis of androstenedione (A4) from cholesterol, by the thecal cell, upon LH stimulation. A4 diffuses across the basement membrane to the granulosa cell where it is converted to estradiol (E2) upon stimulation with FSH. E2 is synthesised in the granulosa cell and not the thecal cell, due to the presence of the aromatase. The specific enzymatic steps which catalyse each reaction in the steroidogenic pathway (depicted by individual purple arrows) are described in detail in Section 1.1.5.

At the beginning of the follicular phase the pool of antral follicles have differing degrees of FSH sensitivity. The follicle with the lowest threshold for FSH stimulation will most quickly up-regulate granulosa cell LHR and aromatase (45). This action culminates in increased sensitivity to gonadotrophins (52), and an exponential increase in estradiol production over the follicular phase (Fig. 1.3). Estradiol has a number of effects. It acts locally at the level of the reproductive tract to promote proliferation of the endometrium in preparation for implantation (53, 54). It also acts peripherally, by negative feedback indirectly to the hypothalamus (55) and directly to the pituitary (56), to dampen FSH secretion. In addition, the granulosa cells of antral follicles also produce large amounts of inhibin which add to the negative feedback effect on FSH secretion from the pituitary (21) (discussed further in Section 1.1.4). Follicles that are less sensitive to FSH will not survive the decline in FSH secretion and undergo atresia. In women, one dominant follicle will remain and continue growing from ~5mm up to ~20mm (45, 47) despite the reduction in circulating FSH. This follicle also has marked aromatase activity (57-59) and further augments estradiol production. Estradiol additionally exerts an intra-ovarian positive feedback loop, inducing the up-regulation of estrogen receptor (ER) in granulosa cells, the effect of which stimulates further estradiol production (21). In conjunction with FSH, estradiol stimulates the

expression of LHR in late antral follicles (21). Gonadotrophin dependency and timing of expression of the gonadotrophin receptors are summarised in Table 1.1.

The growth promoting affects of insulin-like growth factor (IGF) signalling also have a role within the dominant follicle. IGF1 is predominantly produced by the liver in response to stimulation by GH which is secreted by the pituitary (31). In addition, IGFII and some IGF1 is produced by the steroidogenic cells of the ovary (60). IGF1 stimulates granulosa cell proliferation as well as enhancing FSH-induced estradiol secretion (61, 62). A reduction in the levels of IGF binding proteins (IGFBPs) in large preovulatory follicles would lead to enhanced IGF bioavailability and therefore support the continuation of growth and steroid production (63, 64). Furthermore, the blood supply to large follicles is increased, to support enhanced delivery of gonadotrophins to granulosa cells (65). The pro-angiogenic factor, vascular endothelial growth factor (VEGF) stimulates the growth and branching of blood vessels in large follicles and is increased by gonadotrophin-stimulation of granulosa cells of pre-ovulatory follicles of Macaque monkeys (66). In fact, the treatment of Marmoset monkeys with a VEGF antagonist, VEGF-Trap, to block angiogenesis, resulted in the loss of large antral follicles without affecting earlier follicle stages (67).

At around day 12 of the human menstrual cycle, estradiol secretion from the preovulatory follicle reaches a certain threshold and, instead of negative feedback, this triggers a positive feedback mechanism to the hypothalamus and pituitary gland (21). The effect of this is a sustained burst of GnRH neuronal firing and de-granulation of gonadotrophs, resulting in a large surge of LH release from the pituitary (Fig. 1.3). It is this high amplitude peak of LH that triggers, approximately 36 hours later, ovulation and the release of the oocyte, through inflammatory-mediated rupture of the ovarian wall (68, 69). As well as follicular rupture, ovulation is associated with oocyte maturation and luteinisation of granulosa cells. In the oocyte, meiosis I is resumed and completed, there is expulsion of the 1st polar body and meiosis II is entered before being arrested again, leading to cytoplasmic maturation (21). As soon as the LH surge occurs follicular estradiol secretion declines and large amounts of LH-induced progesterone are synthesised, by thecal and granulosa cells (14). This is secondary to an induction of steroidogenic enzymes in the granulosa cells.

Following ovulation, the follicular cells become terminally differentiated and luteinise to form the corpus luteum. The corpus luteum is a highly vascularised glandular structure, in

which the extensive blood supply allows for the delivery of low density lipoprotein to the granulosa lutein cells, promoting progestogenic steroidogenesis (14). The high levels of progesterone secreted during the luteal phase have two roles: to prime the endometrium for possible implantation (53, 54) and, with luteal inhibin and estradiol secretion, to negatively feedback to the hypothalamus to dampen pituitary gonadotrophin secretion and block follicular activity in the ovary (21). FSH concentrations remain low and LH pulsatility is reduced to approximately one every six hours (21). The continuous, albeit slowed, secretion of LH ensures maximal progesterone secretion from the corpus luteum over the luteal phase (Fig. 1.3). If conception and embryo implantation do not occur, the corpus luteum undergoes luteolysis and progesterone levels decline. In the human and higher primates luteal regression is thought to be inherent to the corpus luteum so that the low levels of LH do not promote long-term survival (21). In women, progesterone withdrawal initiates menstruation and the endometrium is sloughed off. This represents day 1 of the next menstrual cycle. The progesterone block on gonadotrophin secretion is removed and tonic LH and FSH secretion is initiated once more to promote antral follicle growth.

1.1.3 The sheep estrous cycle

The sheep, like most other mammalian species including domestic animals and rodents, undergoes reproductive cyclicity termed estrous cycling. Menstruation does not occur in these species as is the case in humans and some primates. The estrous cycle is divided into distinct stages known as estrus, metaestrus, di-estrus and pro-estrus. In sheep the estrous cycle occurs over a 17 day period, in which the follicular phase takes place over the first 2-3 days and there is a pro-longed luteal phase lasting approximately 15 days. During estrus, a period of 24-36 h, the ewe is receptive to the male and will allow mating, and ovulation occurs. The luteal phase is split into metaestrus in which there is formation of the corpus luteum (often corpora lutea) and progesterone levels increase, di-estrus during which progesterone levels are maximal and pro-estrus when the corpus luteum regresses and progesterone levels decline. Most antral follicle growth and development occurs during the luteal phase, since, unlike women, the corpus luteum does not produce inhibin and estradiol as an additional break to gonadotrophin secretion. The final pre-ovulatory stages are completed during the short follicular period (21). With the exception of primordial follicles, the follicle diameters at each developmental stage are much smaller in the sheep than in the human (Table 1.1). Furthermore, in sheep, regression of the corpus luteum is induced by a

luteolytic factor rather than being spontaneous as in humans. The endometrium secretes the prostaglandin, $PGF_{2\alpha}$, as a result of stimulation by oxytocin released by the corpus luteum. The endometrium is sensitised to oxytocin by the up-regulation of oxytocin receptors towards the end of the luteal phase (21). $PGF_{2\alpha}$ travels to the ovary via the uterine vein and ovarian artery where it acts directly on the corpus luteum to promote luteal regression (21).

Much of what is known about the regulation of follicle growth and development by gonadotrophins stems from studies in the sheep. As in humans, antral follicle growth is dependent on the presence of both LH and FSH and while follicles at earlier stages are responsive to gonadotrophins, survival is not dependant on them (70). Hypophysectomy (71), immunosuppression of GnRH (72) or desensitisation of the pituitary by chronic GnRH stimulation (73), ablates the FSH signal and prevents follicle growth in ewes beyond that of 2.5 mm in diameter. In hypogonadotrophic ewes, established through chronic administration of a GnRH analogue, the addition of exogenous gonadotrophins has shown that FSH alone can drive the growth of a pre-ovulatory follicle, however LH alone cannot (73, 74). There is however evidence for the role of LH in the selection of the dominant follicle. Campbell *et al*., using a GnRH-antagonist treated ovine model with a controlled infusion of FSH and LH, showed that withdrawing FSH and LH led to atresia in pre-ovulatory follicles, however, removal of FSH but continued treatment with LH permitted some survival of pre-ovulatory follicles, albeit with an overall reduction in the ovulation rate (75). Furthermore, LH can indirectly influence FSH secretion by increasing estrogen secretion and inhibin production, further promoting survival of the dominant pre-ovulatory follicle (76).

The most striking deviation from the regular cyclicity seen in women is the control of reproductive function by external environmental factors that ensure birth at optimal times of the year. In sheep, HPO activation or suppression is regulated by day-length and thus sheep are seasonal breeders. The switching on of the HPO axis, by initiation of hypothalamic GnRH pulses occurs as a direct response to changing photoperiod, such that shortening day length and lengthening nights in autumn trigger the commencement of estrous cycling. During the hours of darkness the pineal gland, attached to the epithalamus in the brain, secretes a hormone called melatonin, and it is the prolonged period of melatonin secretion that is responsible for hypothalamic activation (21). In springtime, as the nights shorten, so does the duration of melatonin secretion and consequentially estrous cycling terminates. Ewes enter anestrus and are reproductively inactive until the following autumn. Male sheep

are also affected by seasonality, with reduced sperm production and quality as the photoperiod lengthens.

Follicle Stage	Primordial	Primary (pre-antral)	Secondary (antral)	Tertiary (pre-ovulatory)
FSH/LH dependent	–	–	+	+
LHR	–	–	TC +	TC + GC +
FSHR	–	–	GC +	GC +
Size (human)[1]	30 µm	200-400 µm	Small: 2-7 mm Large: 7-16 mm	16-22 mm
Size (sheep)[2]	<60 µm	>60 µm	Small: 1-3 mm Large:3-4 mm	4-8 mm

Table 1.1 Follicular characteristics in the human and sheep. Gonadotrophin dependency, gonadotrophin receptor up-regulation, and follicle size according to follicle stage. [1] (21), [2] the follicle sizes listed for sheep are approximate and are known to vary between breeds (77). TC: thecal cells, GC: granulosa cells.

1.1.4 The role of activin, inhibin and follistatin in reproductive function

Activin and inhibin are members of the transforming growth factor (TGF) β family of signalling molecules. They possess similar structures and are made up of a set of subunits including inhibin α, inhibin βA and inhibin βB (78), which are respectively encoded by the *INHA*, *INHBA* and *INHBB* genes (Fig. 1.5) (79). Dimerisation of these subunits into specific conformations leads to the formation of activin or inhibin proteins with opposing biological effects. Figure 1.5 illustrates the possible subunit conformations required to form each of these signalling proteins. Activin binds to activin type I and type II serine threonine receptor kinases to initiate downstream TGFβ signalling (80). Binding to a type II receptor (ACVR2A and ACVR2B) leads to dimerisation and phosphorylation of a type I receptor (ACVR1, ACVR1B and ACVR1C) (81) and subsequent SMAD protein signalling (82). Inhibin suppresses activin action by binding to and occupying the activin receptor (83, 84). Inhibin can also signal through the TGFβR3 (also known as β-Glycan), which increases the binding affinity of inhibin to activin type II receptors (85). The negative effects of inhibin action can also be augmented by sequestration to secreted binding proteins (86). Follistatin, encoded by

the *FST* gene, is a glycoprotein that binds to both activin and inhibin (87, 88), negating activin signalling although having a negligible effect on inhibin action (88-90).

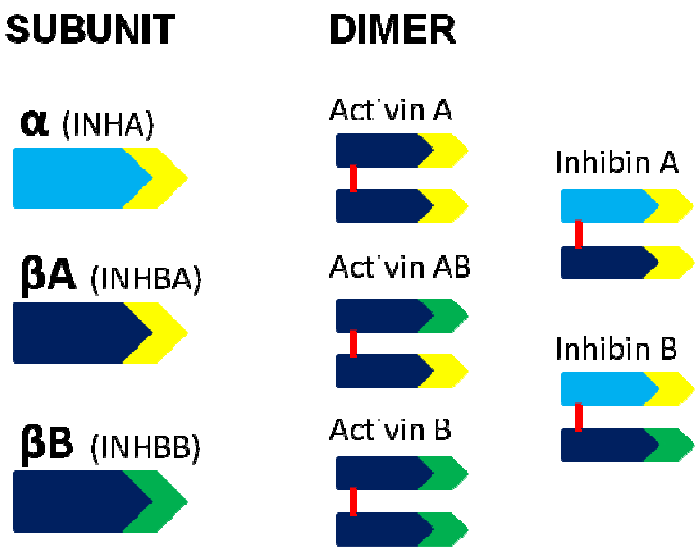

Figure 1.5 The activin and inhibin subunits. The possible conformations of inhibin subunit (α, βA and βB) dimerisation to produce activin A, activin AB, activin B, inhibin A or inhibin B. The gene names for each inhibin subunit are in brackets. Dimers are covalently linked by a disulphide bond (red line).

Activin and inhibin are known to have both endocrine and paracrine roles and are expressed in a range of reproductive and non-reproductive tissues (91). The most commonly studied role for these proteins is their gonadal and extra-gonadal role in reproductive function (92). Both the ovary and the pituitary express activin and inhibin, and these molecules have essential reproductive roles in pituitary function and folliculogenesis (92, 93). One of the major contributions to this system is the respective stimulatory and suppressive effect that activin and inhibin exert on FSH secretion (94, 95). There is differential expression of activins and inhibins. Across the menstrual cycle in women, systemic activin A is found to be raised around the late follicular stage, then falls again until the late luteal/early follicular phase when there is a peak before falling again (96). During the follicular phase circulating inhibin B is elevated, however it is inhibin A that is increased in the luteal phase (97, 98). Free follistatin is not found to vary over the menstrual cycle and is present at low levels in serum, suggesting local action (99, 100). Furthermore, low levels of activin A and activin B are present in serum indicating autocrine or paracrine action, while inhibin may have actions both locally and peripherally (98, 101).

1.1.4.1 The role of activin, inhibin and follistatin in the pituitary

In the pituitary, the local action of activin and follistatin have respective stimulatory and inhibitory effects on the secretion of FSH from the gonadotroph (102), while the endocrine action of gonadal inhibin is also responsible for the suppression of FSH release (103). This inhibin-mediated control of activin signalling appears to be limited to the gonadotroph since inhibin does not reverse the activin-mediated suppression of GH nor ACTH from somatotrophs and corticotrophs, respectively (85, 102). The *in vitro* affects of activin and inhibin on FSH function are well documented. Exposing a rat pituitary cell line to an activin B neutralising antibody suppresses the secretion of FSH (104), as does treatment with follistatin (105). The local pituitary bio-availability of activin has also been demonstrated to be regulated by hypothalamic GnRH secretion. Low amplitude GnRH pulse stimulation of female rats led to an increase in pituitary activin βB (*INHBB*) and FSHβ (*FSHB*) subunit messenger (m) ribonucleic acid (RNA) expression (106), in agreement with human studies showing an increase in FSH synthesis as a result of low frequency luteal phase GnRH pulses, although this *in vivo* mechanism is overcome by strong inhibition by luteal progesterone, estradiol and inhibin (107). Not only does GnRH signalling lead to an up-regulation of activin subunits, activin is required for GnRH-mediated FSH synthesis and has a role in increasing gonadotroph sensitivity to this hormone. *In vitro* studies have shown that activin can target the FSH (108, 109) and GnRH gene promoters (110). While activin can act directly to increase FSH transcription, high levels of activin A can indirectly induce GnRH gene expression thus augmenting downstream FSH release.

1.1.4.2 The role of activin, inhibin and follistatin in the ovary

In the ovary, activin and inhibin act locally to regulate folliculogenesis (92) and ACVR1 and ACVR2 are expressed in granulosa cells, thecal cells and the oocyte (111). Studies have demonstrated the role of activin in follicle growth, differentiation and the selection of large pre-ovulatory follicles *in vitro*. Culture of rat granulosa-oocyte complexes with activin A and insulin led to an increase in granulosa cell number, which could be inhibited by the addition of follistatin (112). Similarly, the inhibitory effects of follistatin were confirmed by the over-expression of follistatin (*FST*) in mice, leading to follicle growth arrest and infertility in adult females (113). Activin A, in the presence of FSH, also led to the formation of large pre-ovulatory-like follicles *in vitro* (112). *In vivo*, the growth affects of activin were

demonstrated in an *INHA* mouse knockout model, in which the lack of inhibin-mediated activin inhibition resulted in extensive granulosa cell proliferation that led to the development of ovarian tumours (114). Furthermore, *ACVR2* knockout resulted in mice with antral stage follicle arrest (115), illustrating the importance of activin for regulated follicle growth and differentiation.

The selection of the pre-ovulatory follicle is enhanced by the numerous roles of activin. Xiao *et al*. reported that activin treatment of rat granulosa cells induced the up-regulation of FSHR, which was reversed by the addition of follistatin (116). Activin also enhanced follicle sensitivity to FSH and LH in these cultures, which resulted in increased aromatase activity (estradiol secretion), as well as progesterone and inhibin production (116). FSH-induced *LHR* induction has also been shown to be stimulated by activin in combination with FSH, in rat granulosa cells (117). Miró *et al.* demonstrated that the precise role of activin in regulating granulosa cell steroidogenesis was stage dependent. The authors cultured rat undifferentiated (-FSH), differentiated (+FSH) and pre-ovulatory (+FSH and LH) granulosa cells with activin A. They found no change in steroid synthesis in undifferentiated cells, however in the presence of FSH, estradiol and progesterone secretion was increased (118). Increased aromatase activity (estradiol secretion) was present in pre-ovulatory cells in response to activin, as well as in differentiated cells, however in the presence of LH progesterone secretion was in fact suppressed (118). In accordance with this, treatment of bovine pre-ovulatory granulosa cells with activin A suppressed progesterone production from these cells (119). These data imply that while activin has a role in stimulating differentiation in the mature follicle, it also prevents premature luteinisation from occurring thus exerting a dual role in inducing and maintaining the folliculogenic phenotype (92).

A major local ovarian role for inhibin is the stimulation of LH-mediated androgen synthesis by thecal cells. *In vitro* inhibin treatment of human thecal cells led to an increase in androstenedione and dehydroepiandrosterone (DHEA) production, an effect which could also be suppressed by treatment with activin A (120). Accordingly rat thecal interstitial cells and thecal implants treated with LH, increased androgen biosynthesis upon the addition of inhibin (121). Inhibin also increased LH-stimulated androstenedione secretion by bovine thecal cells, which was again reversed by activin A treatment (122). The increase in androgens is required as a substrate for aromatase conversion to estradiol within the granulosa cell (123) (Fig. 1.4). As the follicle matures, inhibin secretion from granulosa cells

increases in parallel with an increase in local steroid production. Hillier *et al.* showed that the treatment of human immature follicles with FSH results in an increase in inhibin that is augmented further by the addition of androgens. Furthermore, treating pre-ovulatory thecal cells with LH led to an increase in inhibin synthesis, corresponding to the time when steroidogenesis is high (124).

Therefore, as the follicle matures local activin levels are thought to decrease while levels of inhibin and follistatin rise. Activin maintains the growing, estradiol secreting unluteinised preovulatory granulosa cells, and at the time of the LH surge, activin is withdrawn and inhibin increases, thereby exerting key roles in follicle interactions and maturation. This complex system may be dysregulated in ovarian disorders such as PCOS. In PCOS, a syndrome characterised by the follicular arrest of immature follicles, it has been shown that serum activin A is elevated and follistatin levels are low, which could contribute to this phenotype observed in these patients (125, 126). A decrease in follicular fluid inhibin A and inhibin B subunits have been described in women with PCOS (127), as well as a reduction in the expression of *INHA* and *INHBA* subunit mRNA from PCOS granulosa cells (128). However, peripheral inhibin concentrations have been found to be increased in women with PCOS (129, 130). It is clear that activin and inhibin signalling in the ovary is complex and involves tightly controlled processes that are dependent on the stage of folliculogenesis and the presence of various intra- and extra-ovarian factors, which include IGFs, IGFBPs (131) and also other members of the TGFβ signalling family including growth differentiation factor (GDF) 9 and bone morphogenetic protein (BMP15) (132).

1.1.5 Ovarian steroidogenesis

The ovary is a major source of steroid synthesis in the adult, predominantly secreting estrogens and androgens during the follicular phase of the ovulatory cycle, and progestagens during the luteal phase. The steroidogenic machinery required for the synthesis of the sex steroids is also present during fetal development, with the fetal ovary proposed as a source of steroid production. The steroidogenic pathway takes place within the mitochondria and cytosol of cells and involves a complex series of enzymatic steps converting steroid precursors into various hormones from a primary precursor. Free cytoplasmic cholesterol is transported from the outer to the inner mitochondrial membrane by steroidogenic acute regulatory protein (StAR), a protein present in the mitochondria of steroidogenically active

tissues (133). Various classes of enzymes are then responsible for the sequential conversion of cholesterol and include the cytochrome (CYP) P450 enzymes and the hydroxysteroid dehydrogenases (HSD). CYP enzymes are monooxygenases, hydrolysing and cleaving their steroid substrate (134). Examples of the CYP enzymes include CYP17 (17α-hydroxylase), CYP19 (aromatase) and CYP21 (21-hydroxylase that is expressed in the adrenal), which are microsomal proteins, and CYP11A (P450 side chain cleavage) and CYP11B (11β-hydroxylase that is expressed in the adrenal), which are membrane bound (134). The HSDs are alcohol dehydrogenase reductases which catalytically reduce and oxidise their steroid substrates (134). The CYP enzymes are products of a single gene, however, in contrast, the HSD proteins consist of various gene isoforms that are often species and tissue specific (134). Figure 1.6 illustrates the steroidogenic pathway in the ovary that culminates in the production of progesterone, androgens and estrogens. Below a short description of each of the main enzymes involved in ovarian steroid biosynthesis are provided.

- **CYP11A (P450 side chain cleavage)**

The conversion of cholesterol into pregnenolone by CYP11A is the rate-limiting step of the steroidogenic pathway, acting first in the chain of enzymatic reactions inside the mitochondrial matrix (133). Access to CYP11A is controlled by StAR, which is tightly transcriptionally regulated. CYP11A is well conserved between species and expressed in numerous tissues other than the ovary, including adrenal, placenta and testis (134). Pregnenolone is transported from the mitochondrial matrix and travels to the endoplasmic reticulum whereupon it is converted into a series of steroid products in the presence of the appropriate enzymes.

- **CYP17 (17α-hydroxylase)**

CYP17 is responsible for two successive catalytic reactions leading to the formation of androgenic precursors. Firstly, CYP17 adds a hydroxyl group (hydroxylase) to pregnenolone or progesterone, converting these substrates to the intermediaries 17α-OH pregnenolone and 17α-OH progesterone, respectively. These products are then cleaved by CYP17 (lyase activity) to form DHEA and androstenedione, respectively (134).

- **3β-HSD**

Encoded by the gene HSD3B1 and 2, 3β-HSD1 and 2, respectively (135), are involved in the substrate conversion of intermediate steroids pregnenolone to progesterone, 17α-OH pregnenolone to 17α-OH progesterone and DHEA to androstenedione. In the human, the two isoforms of 3β-HSDs are thought to be differentially expressed, with 3β-HSD1 expression exclusive to the non-classically regulated steroidogenic tissues including mammary tissue, placenta and skin, and 3β-HSD2 expression localised to the gonads and adrenal gland (136, 137).

- **CYP19 (aromatase)**

Aromatase is required for the catalytic conversion of androgenic substrates into estrogenic products. CYP19 reduces androstenedione and testosterone into estrone and estradiol, respectively. As well as being expressed in the ovary (138), CYP19 is expressed abundantly in the human (138), cow, pig and horse placenta (139), however it is not expressed in the placenta of rats and mice (140). There is widespread expression of CYP19 across tissues with notable expression in adipose tissue and bone, and therefore these tissues are alternative sources of estrogen (138).

- **17β-HSD**

There are numerous isozymes of the 17β-HSD protein, which are encoded by HSD17B genes, however they share little homology, possessing differing roles and substrate specificity depending on the tissue type in which they are expressed (134). Type 1 17β-HSD is expressed in the gonad and is thought to have species dependant roles such that the enzyme converts androstenedione into testosterone, or estrone into estradiol, in rodents, however, in humans this activity is restricted to the estrogenic pathway (134). Other isoforms exist including 17β-HSD3 which converts androstenedione into testosterone in the testis (141), and 17β-HSD7 may be responsible for ovarian estradiol synthesis during the luteal phase of the cycle in mice (142, 143) and primates (144).

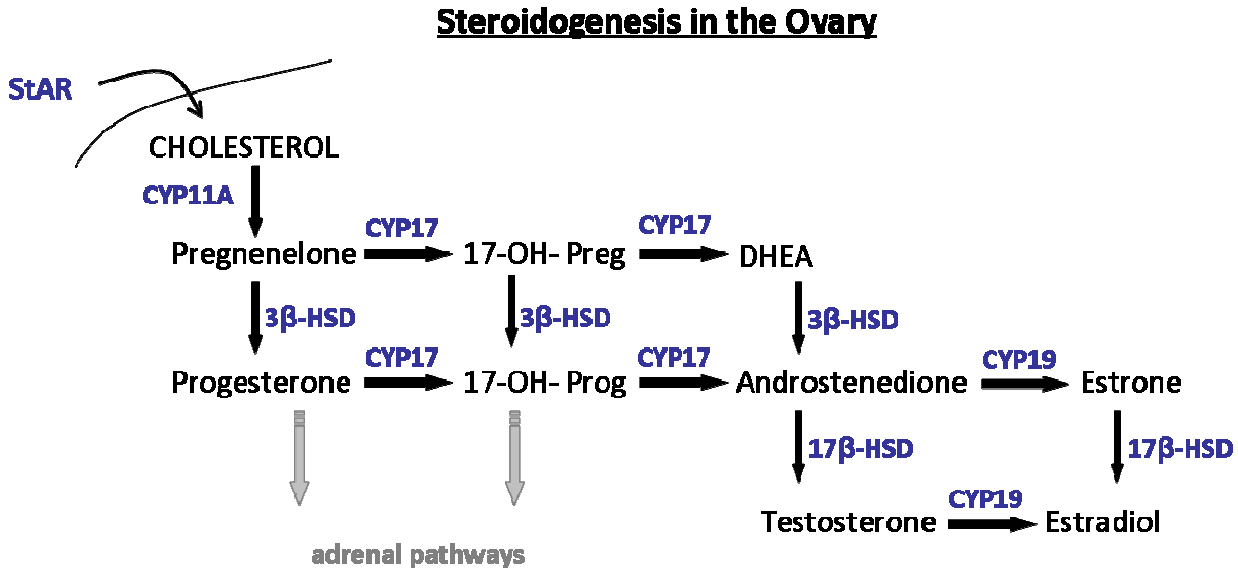

Figure 1.6 The steroidogenic pathway in the ovary. Precursors and products of the steroidogenic pathway are represented in black and the enzymes required for each bio-synthesis step are indicated in blue.

1.1.5.1 The cellular expression of steroidogenic enzymes in the adult ovary across species

In the human ovary, StAR is localised to the thecal cells of pre-ovulatory follicles as well as being present in the corpus luteum in steroidogenic cells of thecal and granulosal origin (145). The expression of StAR in the oocyte throughout follicle development, as well as the expression in thecal cells of late pre-antral follicles, have also been described in the sheep ovary (146). The expression of CYP11A is reported in the granulosa cells and thecal cells of antral or pre-ovulatory follicles in the pig (147) and rat (148), and exclusively in the thecal cells of the human ovarian follicle (149). In the sheep, CYP11A is expressed in thecal cells from the late pre-antral stage onwards, however it is not expressed in granulosa cells until the late antral stage (146). CYP17 is predominantly expressed by the ovarian thecal cell as a means of androgen production and is not thought to be present in granulosa cells of women, sheep, cattle and pigs (149-151). In thecal cells, CYP17 is up-regulated by the early antral stage in the sheep ovary (146). The expression of 3β-HSD may be species specific: immunolocalisation has been observed in the thecal cells of sheep and pigs, but in the granulosa cells of cattle (151). Conversely 3β-HSD has been observed in ovine granulosa cells up to the small pre-antral follicle stage, whereas expression in thecal cells was first

noted in large pre-antral follicles and beyond (146). In the rat, 3β-HSD is expressed in both thecal and granulosa cells of antral and pre-ovulatory follicles (152). The granulosa cells are the site of aromatase expression in the human ovary (138, 150) and aromatase is expressed in granulosa cells of large antral follicles in the sheep (146). In the pig however, CYP19 has been localised to both thecal and granulosa cells of pre-ovulatory follicles (147). There is abundant expression of 17β-HSD1 in granulosa cells of human (153, 154) and rat ovaries (155), which is required for the final conversion step of androstenedione into estradiol via CYP19-mediated estrone conversion (Fig. 1.4 and 5).

1.1.5.2 Steroidogenesis in the fetal ovary across species

In the mouse it is suggested that the fetal ovary is not steroidogenically active. While 3β-HSD can be detected in the mouse fetal ovary, the expression of CYP11A, CYP17 and CYP19 during the time of sexual differentiation is infrequent (156). Similarly, in the rat E18.5 fetal gonad, CYP11A was not detected by immunolocalisation or by an activity assay involving the conversion of tritiated cholesterol into pregnenolone (157). Conversely, the bovine fetal ovary has been demonstrated to be steroidogenically active throughout gestation, with the secretion of androstenedione, estrone and estradiol from cultured fetal ovaries. Estradiol production was found to peak in these ovaries from early to mid-gestation (158). Additionally, active steroidogenesis in the sheep fetal ovary is described. Quirke *et al*. found the presence of estradiol in d35 fetal ovary homogenates and androstenedione and testosterone in d75 fetal ovaries. Furthermore, *StAR*, *CYP11A*, *CYP17* and *CYP19* mRNA were detected in all fetal ovaries by d35 of gestation (159). Interestingly, *HSD3B* transcript was present in the ovine fetal ovary before sexual differentiation and then declined thereafter (159).

1.2 Polycystic Ovary Syndrome – an ovarian, endocrine and metabolic disorder

PCOS is a common disorder affecting between 6-10% of women in their reproductive years (160). It has been recognised for many years but never fully characterised and many aetiological mechanisms are not understood. PCOS occurs in humans, but as this review will discuss, animal models have been developed over the last two decades that have contributed to our basic understanding and have also revealed the complexity of the syndrome.

1.2.1 Clinical features and diagnostic criteria

Amenorrhea, a polycystic ovarian morphology and their association with clinical endocrine abnormalities, such as elevated androgens, have been documented for many years. As far back as 1935, Stein and Leventhal described these features in a subset of women and also commented on the heterogeneity present between individuals (161). Many women first present to their doctor when they are adolescents or young women that are experiencing irregular (oligomenorrhoea) or absent (amenorrhoea) menses, or difficulty in conceiving (162). These symptoms reflect the reproductive element of PCOS for which the syndrome is named. On ultrasound scan, these women are likely to have polycystic ovaries (PCO) which are defined as having more than 12 antral follicles that are between 2-9mm in diameter (Fig. 1.7). The ovarian volume is also likely to be enlarged (163). Rather than being 'cystic' these ovaries are polyfollicular, with an increased number of antral stage follicles present in the periphery of the ovary and a thickened stromal compartment. However, there are many other features of PCOS that are often clinically and biochemically apparent. A major biochemical marker of PCOS is raised androgens which symptomatically result in hirsutism and acne (162). Other hormones are also frequently affected; including elevated levels of LH and an increased LH to FSH ratio (164, 165). Women are often, but not always, clinically obese which combined with the other features of PCOS makes them particularly prone to the development of the metabolic syndrome (162, 166). The accumulation of central fat, a feature that is facilitated by hyperandrogenism is a risk factor for cardiovascular disease, insulin resistance and type II diabetes. Women with PCOS tend to have elevated serum insulin concentrations before and after stimulation with glucose (167, 168). Insulin insensitivity in these women is also independent of body mass index (BMI) and therefore an intrinsic feature of PCOS that is exacerbated by obesity. A further clinical feature is the

presence of acanthosis nigricans, a dark pigmentation that occurs on the folds of skin and is a marker of insulin resistance. It is common in obesity and endocrine disorders including PCOS (169, 170).

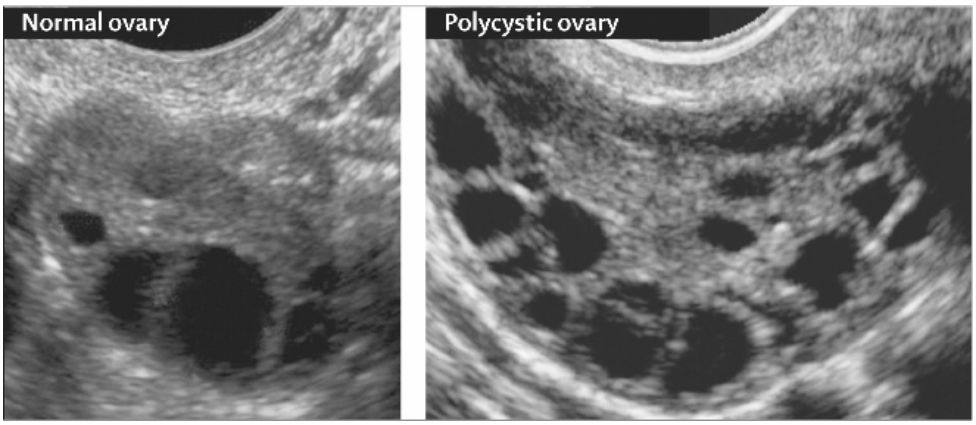

Figure 1.7 An ultrasound scan of a normal and polycystic human ovary.

From Norman *et al.* 2007 (163)

While there are a range of clinical, biochemical and ultrasonographic features of PCOS, they are by no means all inclusive, and this has led to difficulty in the acceptance of standardised criteria used to diagnose this widely occurring and heterogeneous syndrome (171, 172). No one criterion is sufficient to diagnose PCOS and over the last two decades slightly differing guidelines have been published in what should constitute a positive diagnosis. The 1990 National Institute of Health (NIH) guidelines stated that chronic anovulation combined with the clinical or biochemical presence of hyperandrogenism was sufficient to identify PCOS (173). In 2003, the Rotterdam consensus was published that expanded on the NIH guidelines and included the presence of PCO as a diagnostic criterion for PCOS (174). This resulted in the possible definition of four separate but closely related phenotypes of PCOS and reflected more on the complexity and heterogeneity of the syndrome (Table 1.2). It has been estimated that, of the women that are diagnosed based on the Rotterdam criteria, approximately two thirds have 'severe PCOS' combining all of the features of PCOS, whereas less than 10% will possess these features without PCO (175). The remaining women are split between 'mild PCOS' or 'ovulatory PCOS' phenotypes (175). These criteria therefore allow for a more broad definition of the different types or severity of PCOS. Both sets of guidelines are careful to rule out underlying illnesses such as congenital adrenal hyperplasia, androgen

secreting tumours and Cushing's syndrome as potential sources of androgen excess that are unrelated to PCOS, but may initially share common features (163).

PCOS Traits	Mild PCOS	Ovulatory PCOS	Severe PCOS	Anovulation/ hyperandrogenism*
PCO	Yes	Yes	Yes	No
Androgens	↑	↑↑	↑↑	↑↑
Ovulation	Irregular	Normal	Irregular	Irregular
Insulin	Normal	↑	↑↑	↑↑

Table 1.2 The criteria for the diagnosis of PCOS based on the 2003 Rotterdam consensus. This set of criteria results in a broad spectrum of severity of PCOS and highlights the heterogeneity of the disorder. * Women falling into this category would be classified as PCOS under the guidelines published in1990 by the NIH.

Adapted from Norman *et al.* 2007 (163)

Unsurprisingly, the introduction of the broader Rotterdam criteria in the diagnosis of PCOS has resulted in a higher overall prevalence of women with PCOS (176). Additionally, the increasing level of obesity and diabetes in Western countries, if not an underlying cause, certainly augments the severity of PCOS. Obesity and anovulation have long been known to be associated with one another (177). This is especially proven by the spontaneous re-instigation of normal ovarian cyclicity when women reduce their dietary intake and assume regular exercise (178-180). Further complexity is added when ethnicity is taken into account: the prevalence of PCOS is altered between different ethnic populations, and is increased in populations in which insulin resistance and the metabolic syndrome are common (181). Studies have shown that there is an increased prevalence of PCOS in Hispanic Americans opposed to white or black Americans (182). The prevalence of PCOS in Asian women from the Indian sub-continent was also found to be very high (183).

1.2.2 Current management and treatment strategy

It is widely agreed that the initial treatment to improve reproductive function in overweight patients should be weight-loss (163, 179, 184, 185). Studies have shown that a 2-5% decrease in body weight, particularly in central fat, can improve the metabolic and reproductive characteristics of PCOS (186). Furthermore, an 11% reduction in body fat was associated with vastly improved insulin sensitivity and lowered basal levels of insulin, and reduced LH concentrations (187). In women who do not wish to become pregnant, treatment with an oral contraceptive pill is a strategy to regulate menstrual bleeding and also to suppress androgens, thereby improving some of the clinical manifestations of this hormone, including hirsutism and acne (163). There are a range of options for treatment to induce ovulatory cycles and increase the chances of conception. These include treatment with anti-estrogens such as clomiphene citrate, which has the effect of dampening of estrogen negative feedback and increasing the secretion of gonadotrophins, particularly FSH (188). This treatment has been shown to improve ovulation rate and increase the chances of conception in young women (188, 189), however it has also been linked to multiple pregnancy and miscarriage (184, 190). Not all women are however responsive to these drugs (191) and therefore other ways of reducing estrogen concentrations, including treatment with aromatase inhibitors such as letrozole (192), or antagonising steroid biosynthesis by inhibiting 17α-hydroxylase (CYP17) (193), can be used in conjunction with drugs such as clomiphene. Another strategy is to treat with exogenous FSH, which can successfully restore ovarian cyclicity (194).

Other ways to reduce the androgen burden include the use of the synthetic glucocorticoid, dexamethasone, which suppresses adrenal androgen production can also improve ovulation and pregnancy rate when combined with clomiphene (195). Anti-androgens combined with the oral contraceptive pill can also be used to treat acne and hirsutism. Both cyproterone acetate (an androgen receptor blocker and progestagen) and finasteride (a 5α-reductase inhibitor, which blocks the synthesis of dehydrotestosterone [DHT]) can be used (196). Flutamide is anti-androgen that has been shown to be beneficial in that it can lower androgens, decrease visceral fat and cholesterol and improve hirsutism (197-201).

To combat the metabolic characteristics of PCOS, aside from the obvious and essential changes to lifestyle, drugs are commonly prescribed to improve insulin sensitivity. One of the most widely used drugs is the biguanide, metformin, which reduces the hyperglycaemic state by suppressing hepatic gluconeogenesis thereby lowering glucose production by the liver, and stimulating glucose uptake by peripheral tissues, thus improving insulin resistance (202). Treatment with metformin reduces fasting insulin levels, lowers concentrations of LH and androgens and can induce ovulation in PCOS (203-206). Combining metformin with clomiphene treatment has proven to be successful in some but not all patients (207-210), although it is suggested that clomiphene treatment is overall more effective the metformin (211). The thiazolidinediones including troglitazone, pioglitazone and rosiglitazone, are insulin sensitising drugs that have also been used to treat PCOS. Treatment with these drugs have been shown to reduce androgen levels, induce ovulation and improve insulin resistance (212-216). While the use of the thiazolidinediones have had clear benefits to the PCOS phenotype in clinical trials there has since been concern over their safety and these drugs are no longer used to treat PCOS (217).

Each patient must be individually assessed to tailor the most appropriate treatment to their specific needs. Not all patients will respond particularly well to drugs and treatment is largely symptomatic. In many cases using anti-estrogens or anti-androgens in conjunction with insulin sensitisers and a strict weight loss programme can be the most beneficial (179, 184, 185, 197, 207, 208). In some patients these treatments are extremely successful leading to the induction of ovulatory cycles and improved metabolic function, however in others, current treatment is not adequate. This emphasises the complexity and variation in this disorder that can make PCOS particularly difficult to treat.

1.2.3 Heritability of PCOS

PCOS is a heritable trait that can be passed down the generations from mother to daughter and features of PCOS including hirsutism and metabolic traits are often present in relatives of women with PCOS (218). In fact, sisters of women with PCOS, who have PCO themselves, have fewer but comparable biochemical traits compared to their sisters (219). One of the major indications that PCOS is genetically inherited is the finding, in a large study of Dutch twins, that monozygotic twins shared twice as much resembling features of PCOS features than dizygotic twins (220). Other twin studies have highlighted the

heterogeneity between individuals proposing that a number of genes may be involved in the inheritance of PCOS (221). In addition it is known that male siblings or sons of women with PCOS also have higher risks of the metabolic implications associated with PCOS (222-224) pointing towards genetic involvement. While a lot of research has gone into the search for a gene or set of genes that are responsible for the heritable features of this syndrome this has not been very successful, although a number of candidate genes are proposed to be involved (225). It is now emerging that the more likely mode of inheritance of PCOS is through post-transcriptional epigenetic alterations to the genome that alter the expression of certain genes in offspring. These changes could be programmed by the maternal and/or fetal environment, and it is possible that this, in combination with the inheritance of PCOS susceptibility genes, could result in the highly variable and heterogeneous nature of the syndrome (221). Furthermore, in the event of obesity the susceptibility to PCOS traits are more pronounced, which may be additive or a result of altered gene expression, or even a combination of both (225). The potential modes of transfer of these traits are discussed below.

1.2.3.1 Candidate genes associated with PCOS

A range of genes have been implicated in PCOS that affect steroidogenesis and metabolism (225, 226), although the poor reproducibility and small sample size of many of these studies has been limiting (225, 227, 228). A promising study, published by Barber *et al.*, found that variants of the fat mass and obesity-associated (*FTO*) gene, which is associated with insulin resistance, obesity and type II diabetes (229-231), were linked to PCOS even after correcting for BMI, however, there was no association with increasing androgenaemia (232). Other genes that may be associated with PCOS and have a role in insulin insensitivity include the peroxisome proliferator-activated receptors (233) and the insulin receptor (234). While one study found a mild association between the variable number of tandem repeats region of the insulin gene that is responsible for regulating insulin secretion, with PCOS susceptibility (235), others did not (236, 237).

Potential gene candidates for PCOS include genes involved in steroid biosynthesis. There are varying and inconsistent reports for the association of PCOS with the presence of specific alleles of the *CYP11A* gene (238, 239). A link between PCOS and polymorphisms of the *5α-reductase* gene have also been identified (240). Studies have shown that 5α-reductase, which

is involved in conversion of testosterone into its more potent metabolite DHT and promoter of adrenal androgen output, may have implications for the androgenecity observed in PCOS (241-243). Another mechanism to alter circulating androgen levels in PCOS would be via the aromatase enzyme, encoded by *CYP19*. However studies in this regard are once again inconsistent (239, 244). Although the *CYP17* gene is a good candidate for linkage with PCOS, the presence of specific alleles has not been associated with PCOS (245-247), however, some have been linked to BMI and insulin resistance (247).

Other correlations with PCOS have been found in patients that carry a specific allele of the extracellular matrix glycoprotein, fibrillin 3 (*FBN3*) gene, which has also been associated with increased fasting insulin and insulin resistance (248-250). Since the fibrillins can also regulate members of the TGFβ family (251, 252), which have roles in normal and pathophysiological ovarian function (253-256), the *FBN3* gene became a gene widely researched for its link with PCOS. In addition, a recent study found that in ovaries from women with PCOS, the FBN3 protein was reduced or absent in primordial to primary follicles when compared to normal ovaries (257). This implies a role for this protein in folliculogenesis and links it to circumstances when these processes are disordered.

1.2.3.2 Epigenetic transfer of PCOS traits

The epigenetic modulation of gene expression during development and post-natally confers the plasticity that is crucial to allow adaptation to the external environment (258). Perturbations in this system however can lead to the pre-disposition and onset of adult chronic diseases (259, 260). The Barker hypothesis, coined in 1990, postulated that exposure of a fetus to environmental insults at critical periods of development could programme long term changes in gene expression that manifest in adulthood (261, 262). This mode of epigenetic inheritance may therefore be transferred from mother to fetus through nutrition, stress, hormonal imbalances, exposure to environmental toxins and so forth that could have adverse effects on fetal development (263). There are numerous studies that highlight this programming phenomenon: one of the most cited being the long-term metabolic effects and cardiovascular disease, prevalent in offspring of women that were pregnant during the Dutch hunger winter in 1944-45 (264, 265). The low caloric intake and undernutrition during pregnancy resulted in a cohort of people that were born with low birth weights and were susceptible to diabetes, pulmonary disease and cancers in adult life, which was particularly

prevalent if starvation occurred during the third trimester of pregnancy (265, 266). Interestingly, obesity was more common in those individuals whose mothers were starved during early pregnancy (267) and early exposure to undernutrition has also been associated with schizophrenia and personality disorders (268, 269). Other studies have indicated that fetal adaptation to an unfavourable environment *in utero* can result in poor sensitivity to a plentiful post-natal environment (270, 271). This is commonly reflected by rapid post-natal catch-up growth which itself has been associated with the onset of obesity, diabetes and cardiovascular disease (270, 272, 273). In addition to nutritional stress, maternal emotional stress, anxiety or depression can have adverse effects on fetal neurodevelopment (274). For example, assessment of infants of pregnant women in New York at the time of the 9/11 attacks, found that they displayed cognitive and behavioural impairments at 9 months of age (275). Fetal exposure to the stress hormone cortisol has been directly shown to lead to poor cognitive development in the infant which is confounded by insecure maternal attachment revealing both the prenatal and post-natal impact of anxiety on long-term development (276).

Animal studies have been particularly revealing in research of the cognitive, behavioural and metabolic aspects of fetal programming. The administration of a synthetic cortisol, dexamethasone, to pregnant rats leads to low birth weight in offspring, hypertension, obesity and type II diabetes (277-280). Undernutrition in sheep during early to mid-gestation results in increased adiposity in offspring (281), abnormal growth of the liver in adults (282) and has even been shown to programme an increased response to emotional stressors and differential cognitive responses (283). Late gestational undernutrition in the sheep leads to perturbed insulin-glucose homeostasis in adult offspring (284, 285). Studies of rodent programming models have shown that these traits can also be transferred to subsequent generations (286-288), demonstrating the inter-generational heritability that can occur as a result of adaptation to the *in utero* environment.

There is evidence that humans are susceptible to epigenetic programming throughout life (258), however particularly sensitive periods during fetal or early life are vulnerable to adverse alterations to the environment (263). Widespread epigenetic changes are known to occur in the fetus during early embryogenesis and gametogenesis as this is crucial for the maintenance of cell lineage (260). There are various forms of epigenetic modifications that can control gene expression (Fig. 1.8). One of the most studied is deoxyribonucleic acid (DNA) methylation in which biochemical changes are applied to DNA to negatively regulate

transcription. This is a stable change in transcriptional control that is passed on during cell division (289).

The majority of the genome is thought to be methylated to prevent aberrant gene expression (290). This genetic 'mark' occurs on cytosine (C) and guanine (G) dinucleotide repeats that are termed CpG sites (the 'p' referring to the linking phosphate bond). DNA methyltransferases are responsible for the catalytic transfer of methyl groups to the 5' position of cytosine (289) and thereby maintain gene expression patterns. Areas abundant in CpGs are called CpG islands and these are predominantly located in gene promoters but can be situated in downstream transcriptional regions (289). The binding of the methyl group confers transcriptional silence that is mediated by changes in chromatin structure through the recruitment of histone deacetylases (291). The removal of histone groups from lysine residues on the nucleosome by histone deacetylases renders the gene less transcriptionally active (Fig. 1.8) due to decreased binding of transcription factors and a more closed chromatin structure (259, 291). Hypomethylated CpG islands are generally found on promoters of genes in which transcription is constitutively active (289). An additional source of epigenetic control that is rapidly emerging is the role of small non-coding micro (mi)RNAs (Fig. 1 8). These are highly conserved across species and are approximately 22 nucleotides in length (292). These short sequences bind to 3' complementary sequences of target genes, dampening protein expression by preventing translation or tagging the mRNA sequence for degradation (293).

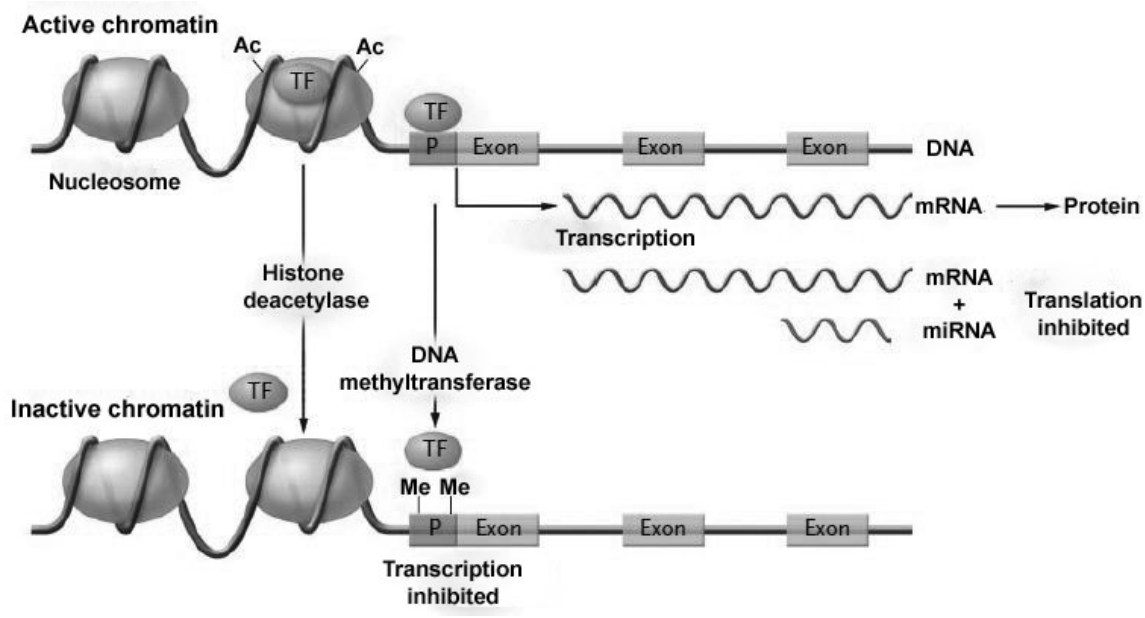

Figure 1.8 Methods of epigenetic regulation of gene transcription. The cartoon illustrates the normal chromatin structure and binding of transcription factors (TF) to DNA promoting gene expression. The action of histone deacetylases and DNA methyltransferases results in modification to the chromatin structure and prevention of TF binding to the promoter (P) region of the gene, thus suppressing transcription. An additional source of epigenetic control is the binding of specific microRNAs to the 3' end of the mRNA sequences, inhibiting protein expression.

From Gluckman *et al.* 2008 (259)

1.2.4 The development of animal PCOS models

Given the lack of success in identifying candidate gene(s) to explain the heritability of PCOS, it is now believed that the epigenetic processes described, may be involved in the early programming of these reproductive and metabolic traits. Without being able to target specific genes, the development of animal models of PCOS would be challenging, however, there are numerous rodent (294, 295), non-human primate (296-298) and ovine (299-301) models available. The development of a model with PCOS-like traits occurred inadvertently through studies that were concerned with the masculinisation effects of prenatal androgens in non-human primates. Following behavioural studies in young offspring, animals were retained and allowed to mature into adulthood. It was in these individuals that the first observations of features reminiscent of PCOS were noted, including hyperandrogenism, PCO and anovulation (296, 298).

If increased androgens are the basis for epigenetic programming of PCOS-like traits in humans, then the question arises as to the source of this steroid during pregnancy. It is possible that the hyperandrogenic state of a mother with PCOS could transfer sex steroids to the fetus (302). However, long-term studies have indicated that the level of circulating maternal androgens during mid- or late-pregnancy have no influence on the development of PCOS in adolescent daughters (303). Furthermore, in instances where maternal androgens are particularly high, due to an androgen secreting tumour, virilisation does not occur in female fetuses suggesting that the placenta is an adequate barrier to androgens (304). Nevertheless, increased androgens could result in placental modifications that then go onto mediate programming effects (305).

It is mostly argued that the fetus or the fetal environment is the source of increased androgens. In the fetus, androgens may be elevated through overactive ovarian or adrenal steroidogenesis. PCOS is also more common in women that have congenital adrenal hyperplasia (306), in which, due to a mutation in the *CYP21* gene, steroid precursors are shunted into the androgen biosynthesis pathway. The fetal exposure of these women to increased adrenal androgens results in PCOS characteristics, however, these phenotypes are severe in that these women are virilised (307), which is not a feature of PCOS. There is some suggestion that female fetuses that share the womb with a male twin could be exposed to increased androgens (308). In litter bearing species such as rodents and pigs there is evidence

that female offspring that have intrauterine positions between two males are born with masculinised morphological and behavioural traits (309). In female human fetuses, the effect of elevated prenatal androgens due to the presence of a male co-twin is not really known; however, there is some associative evidence for programming effects. Studies have observed the masculinisation of auditory development (310), dental crown size (311) and brain volume (312) in females from female-male twin sets. Reproductive fitness was reported to be affected in females that were opposite sex twins, measured by reduced fecundity (313), however, other studies have not identified this association (314, 315).

The relationship between PCOS in adulthood and elevated *in utero* sex steroids may be causative or associative, and whether the altered fetal environment stems from maternal or fetal sources, is not known. The development of animal models of PCOS have however provided insight into the fetal origins of this disorder, and in parallel with human studies, have contributed to our current understanding of the pathways that might lead to the ovarian, endocrine, adrenal and metabolic perturbations present in PCOS. The remainder of this review will discuss the findings from human and animal studies, which although can be complimentary, also highlight the caveats, inconsistencies and challenges to researching PCOS.

1.3 The ovary and PCOS

1.3.1 Ovarian morphology and follicular growth in PCOS

The polyfollicular phenotype, a striking characteristic of PCOS, is associated with anovulation and acyclicity and a disrupted hormonal profile in women (316). Studies of ovary biopsies from women with PCOS have highlighted aberrant follicle growth trajectories that might account for the increased numbers of small follicles present in these ovaries. In fact Webber *et al.* reported that in women with anovulatory PCOS there was an increased density of pre-antral follicles, particularly in the primary follicle pool. In both ovulatory and anovulatory PCOS the proportion of primary follicles increased and there was a corresponding decrease in the proportion of primordial follicles present (317). The enhanced number of growing primary follicles was speculated to be due to either an increase in the pool of primordial follicles or a decline in the rate of follicle regression (317). Maciel *et al.* also reported an increase in the number of growing primary follicles present in the ovaries of women with PCOS, however, they did not find an alteration in the number of primordial follicles present, suggesting that the primordial reserve is not greater nor is there increased activation to transitional follicles. These authors found a greater number of follicles in PCOS compared to control ovaries at each follicle stage, without an increase in atresia, suggesting that the follicular growth trajectory is slowed (318).

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Unlike the study by Maciel *et al*. other studies have found that parallel to the increase in total number of small growing follicles, there was an alteration in the proportions of follicles at each developmental stage (317). Webber *et al.* also showed that there was a reduced loss of preantral follicles in cultured ovarian slices suggesting that atresia may in fact be reduced in PCOS (319). However, prior to culture, the proportion of healthy to atretic follicles between PCOS and control patients was not actually different (319). Discrepancies between studies may be related to the high level of heterogeneity present in individuals with PCOS, as well as slightly differing criteria for the diagnosis of the syndrome. Furthermore, separating women with PCOS into ovulatory and anovulatory cohorts for these types of studies is essential.

Alterations in the level of follicular GDF9 have been proposed to have a role in the perturbation of follicle growth in PCOS ovaries. GDF9 and BMP15 are TGFβ oocyte-

derived factors (320, 321) that are essential for follicular growth. In mouse knockout studies, *GDF9* -/- blocks the growth of follicles at the primary stage affecting thecal, granulosa and oocyte function (322). The importance of BMP15 has been demonstrated in the Inverdale breed of sheep that carry a natural mutation in the *BMP15* gene (323, 324). Sheep with a double mutation in *BMP15* (FecXI/FecXI) have no follicular growth past the primary stage, with normal growth and development up until that point (253). Interestingly this effect is dose-dependent: a heterozygous mutant carrying one normal *BMP15* allele has an increased ovulation and fecundity rate (324). Teixeira Filho *et al.* found that there was decreased oocyte *GDF9*, but not *BMP15* expression, assessed by *in situ* hybridisation, in ovaries from women with PCOS and PCO when compared to control subjects. This decline in *GDF9* occurred in follicles of all developmental stages and implicates the loss of this oocyte-derived growth factor in the aetiology of arrested or slowed follicular growth (325).

Anti-müllerian hormone (AMH), also a member of the TGFβ superfamily, is another factor proposed as potential candidate for follicle growth irregularities in PCOS. AMH is derived from granulosa cells (326) and regulates the entry of follicles into the growing phase. In mice, *AMH* -/- results in an ovarian phenotype similar to that described in ovaries of women with PCOS (317), in which there is a decline in primordial follicle number and a reciprocal increase in growing primary follicles (327). Conversely, culturing mouse ovaries with AMH leads to a reduced proportion of primary follicles, suggesting that AMH inhibits primordial follicle activation (328). Stubbs *et al.* investigated the link between AMH and follicle type and growth in PCOS and non-PCOS ovaries. They found that fewer primordial and transitional follicles were positive for AMH immunostaining in anovulatory PCOS compared to control and ovulatory PCOS ovaries, and that the AMH negative follicles had fewer granulosa cells (329). This study provides evidence that the loss of the AMH signal may have a role in the activation of primordial follicles but AMH may also have a role in granulosa cell proliferation throughout development. Another study by Stubbs *et al.* found that granulosa cell proliferation in preantral follicles was in fact increased in anovulatory PCOS compared to control and ovulatory PCOS ovaries (330). Due to the increased number of preantral follicles present in PCOS, the levels of AMH are increased (331), therefore this could be exerting a stimulatory effect on granulosa cell mitogenesis. Raised levels of ovarian AMH are not thought to effect peripheral primordial follicle development since these follicles do not possess a blood supply (19) and the cortical area is generally avascular (316).

1.3.1.1 The effect of the prenatal hormonal environment on ovarian morphology and follicular growth

Prenatal androgenisation studies in sheep have shown that ovarian morphology and follicular dynamics are disrupted during fetal life. Exposure to testosterone from day (d)30-90 of a 147 day gestation, led to an overall decrease in the number of follicles in the d140 ovary suggesting an effect on ovarian reserve (332). Steckler *et al.* showed that the proportion of follicles grouped by stage was also altered so that there was a decrease in the primordial follicle number and a reciprocal increase in follicles beyond the primordial stage, indicating increased and premature follicular recruitment (332). Similarly, Forsdike *et al.* found increased primary follicle number at the expense of primordial follicle number in 8 month old d30-90 prenatally androgenised lambs (333) in line with studies the human PCOS ovaries (317, 318), although by 20 months this effect was not observed as more follicles were growing in the control cohort (333). In adult d30-90 prenatally androgenised offspring, the tracking of ovarian follicles with the use of ultrasound revealed that prenatal testosterone programmed the failure of large follicles to regress (334). This is in agreement with human studies that show a reduction of follicular atresia in PCOS ovaries (319). This follicular persistence is speculated to be programmed by estrogen since prenatal treatment with DHT from d30-90 did not result in failure of regression (335). Interestingly, DHT, and not testosterone exposure, resulted in an increased number of small follicles in adult ovaries on ultrasound scan (335).

1.3.1.2 The effect of the adult hormonal environment on ovarian morphology and follicular growth

In addition to a programmed or epigenetic origin of the ovarian phenotype in PCOS, the consequential altered hormonal environment that the ovary is subjected to is also thought to contribute to the progression of the syndrome. The hyperandrogenaemia present in women with PCOS, partly caused by increased ovarian synthesis (Section 1.3.2), may also directly affect follicle growth. Vendola *et al.* treated adult normal-cycling Rhesus monkeys with testosterone or DHT for a short period and found that this acute treatment led to an increase in the number of preantral and small antral follicles compared to control ovaries. Thecal cell number and width was increased and granulosa and thecal cell immunostaining for the proliferative marker Ki67, showed an increase in proliferation following both testosterone

and DHT treatments. There was no effect on follicular atresia (336). These treatments also resulted in a significant increase in *IGF1* mRNA in granulosa and thecal cells of preantral and small antral follicles as well as an up-regulation of *IGFR1* mRNA, particularly in thecal cells (337). This is significant given the role of the IGF signalling system in follicle growth. In mice, *IGF1* -/- results in arrested follicle growth due to the suppression of granulosa cell *FSHR* expression, an effect which could be rescued by the addition of exogenous IGF1 treatment (338). Unsurprisingly, *IGF1* is found to be selectively expressed in follicles that are proliferating, and is switched off in luteinised follicles (339). The fact that the changes in gene expression in the studies by Vendola *et al.* were observed after both testosterone and DHT treatment indicate an androgenic signalling effect (336, 337).

In addition, a study from the same laboratory investigated the expression of AR in the adult Rhesus monkey ovary following short-term testosterone treatment. Weil *et al.* found that this treatment resulted in an up-regulation of *AR* mRNA in granulosa cells and a down-regulation in theca cells and stroma from ovaries of testosterone treated animals. Moreover there was increased granulosa cell proliferation and decreased apoptosis in those follicles with more abundant *AR* (340), providing further evidence that increased follicle number and proliferation are androgenically regulated (336, 340). The same group also went on to show that *AR* and *FSHR* mRNAs are co-localised in healthy, growing follicles and short-term testosterone treatment also up-regulated granulosa cell *FSHR* (341) indicating that androgens indirectly promote follicle growth through enhancement of follicle sensitivity to FSH, possibly involving the up-regulation of follicular IGF1 (337). Although these animals were not subject to a chronic or prolonged period of adult androgen exposure, these studies highlight the effects of the local steroid environment in perturbing ovarian function, which are separate to the intrinsic effects programmed by androgens during fetal life. The evidence from the studies in the prenatally androgenised sheep (332-335) and that of Vendola *et al.* and Weir *et al.*, suggest that the follicular dysregulation in women with PCOS (317-319, 329, 330) may be both epigenetic as well as a product of the additive effects of androgens in adult life.

Women with PCOS also tend to be hyperinsulinaemic: a product in part of increased central adiposity and the associated development of insulin resistance. The effects of increased insulin, which can directly target the ovary, may also contribute to the growth arrested phenotype of the PCOS follicle. It is known that insulin targets the granulosa cell via the

insulin receptor rather than the insulin-like growth factor 1 receptor (IGFR1) (342), and insulin treatment of cultured granulosa cells from PCOS patients leads to increased LH stimulated estradiol and progesterone production (343). These data imply that elevated insulin, common in PCOS, would drive early differentiation of granulosa cells and arrest follicle growth and are discussed further in Section 1.6.

1.3.2 Ovarian steroidogenic output in PCOS

Gilling-Smith *et al.* demonstrated the increased ability of thecal cells to produce androgens in PCOS. Androstenedione secretion was significantly increased in cultured PCOS thecal cells compared to controls, both basally and following LH stimulation (344). Progesterone production was also enhanced in the PCOS thecal cells, which was speculated to contribute to increased androstenedione levels through conversion by steroidogenic enzymes (344). This finding has additionally been demonstrated in thecal cells over multiple culture passages implying that this is a stable and intrinsic phenotype (345). Evidence for the global up-regulation of thecal steroid synthesis in PCOS is also provided by the finding that genes involved in the steroidogenic pathway, including *CYP11A* and *CYP17* are up-regulated in PCOS theca, as well enhanced enzyme activity of *CYP17*, *HSD3B* and *HSD17B* (345).

Furthermore, steroid output from granulosa cells are also disrupted in PCOS which is dependent on the ovulatory status of the individual (316). Willis *et al.* showed that normal and ovulatory PCOS granulosa cells respond to FSH and LH in much the same way, by increasing estradiol and progesterone production, with only large follicles responding to LH. However, the authors found that in anovulatory PCOS, not only was the response to LH augmented in terms of estradiol and progesterone secretion, but some small follicles also responded to LH (346). This latter observation could imply premature differentiation of granulosa cells that could contribute to the follicle growth arrest associated with suppressed FSH secretion (316).

In the ovary insulin signalling also has a role in the control of steroid synthesis. Rice *et al.* showed that while insulin signalling in terms of glucose uptake and metabolism is impaired in anovulatory PCOS granulosa-lutein cells, steroidogenesis in the form of progesterone secretion is unaffected (347). This is thought to be due to alternate cellular downstream

insulin signalling pathways relaying the metabolic and steroidogenic responses to insulin. In this regard it is possible that a hyperinsulinaemic state, common in PCOS, would enhance granulosa cell steroid production in these individuals. Indeed, it has been shown that treatment of granulosa cells with insulin resulted in a significant increase in estradiol and progesterone secretion in subjects with PCOS (343).

1.4 Endocrine disruption in PCOS models

Since the early studies investigating the behavioural impact of prenatal testosterone on the offspring of non-human primates (348, 349), many studies have utilised this model as an approach to study reproductive dysfunction and PCOS in a range of animal models. The hormonal and ovarian features of this model, reminiscent of the common characteristics of PCOS have been well described in such studies. Endocrine disruption including LH hypersecretion and increased LH pulse frequency, have been observed in prenatally androgenised non-human primates (350, 351), sheep (299, 352) and perinatally androgenised mice (295) and rats (294). Numerous studies across a range of altricial and precocial animal species find that prenatal androgens have mixed effects on the timing of puberty, adult ovarian cyclicity, LH hypersecretion and increased LH pulse frequency and the ability to produce an LH surge (350). While timing of treatment, dose, type of androgen administered and species difference may account for some disparity, it is clear that *in utero* androgen exposure of a female fetus has a universal impact on reproductive fitness (350). In the sheep, as reviewed in detail below, administration of prenatal testosterone from d30-90 leads to a polyfollicular phenotype (301), perturbations in the timing of puberty (353, 354) and acyclicity (355), as well as LH hypersecretion and increased LH pulse frequency (299, 352). This phenotype has also been observed in other species. In non-human primates early and late prenatally testosterone treated offspring exhibited polyfollicular ovaries (356), anovulation (350, 356) and delayed puberty (357). LH hyper-secretion was however restricted to those animals treated during the early gestational period (358).

In altricial species including mice and rats, testosterone treatment around the perinatal period corresponds to the developmental stage during mid-gestation in many precocial species, such as non-human primates and sheep. Early neonatal androgen treatment of mice from postnatal

day (PND) 1-3 leads to acyclicity in adults (359). Prenatal treatment of dams with DHT resulted in LH hypersecretion and an increased GnRH pulse frequency in adult offspring (295). In rats, testosterone treatment at PND 1 delays puberty in addition to rendering animals acyclic (360, 361). Interestingly, postponing treatment until PND 5 results in the advancement of puberty and acyclicity in the rat (361). Additionally, late prenatal DHT treatment of dams resulted in a lack of inducible LH surge and an increased LH pulse frequency (294). Similar to studies in non-human primates and sheep, the ovaries of prenatally androgenised mice and rats display the characteristic polyfollicular phenotype. In rats, PCO and acyclicity has been induced by the treatment of adult animals with the estrogenic compound, estradiol valerate (362, 363), demonstrating a role for other steroids in the programming of these features. The pre-pubertal treatment of rats with testosterone or DHT also results in an adult PCO phenotype (364, 365). Due to wealth of literature concerning the effects of prenatal androgens on gonadotrophin dynamics, only touched upon here, a detailed overview will be restricted to the studies concerning the sheep PCOS model as this is the species studied in this thesis.

1.4.1 Gonadotrophin dynamics in the prenatally androgenised sheep

During development there is a critical period for masculinisation of the brain. In males, testosterone, produced by the developing testes, drives this programming so that the central nervous system is organised in a male-specific way (366-368). This directs the sequence of events controlling neuroendocrine and reproductive function in pre-pubertal and adult animals. In sheep, sexual differentiation is thought to occur between d30-100 (369). The specific periods for the programming of different types of reproductive function are however thought to be distinct. More than 30 years ago, Clarke *et al*. demonstrated that early testosterone treatment (d30-80) led to a severe masculinised external genital phenotype in female offspring, whereas administering testosterone at a later stage of gestation (d50-100) led to a less severe or normal genital phenotype (370). Male behavioural traits were however programmed in testosterone treated animals regardless of the gestational period of exposure, with aggressive male-like behaviour observed in female animals (370). These ewes displayed mounting behaviour and also adopted the standing male urination posture (370).

In the male sheep puberty begins at approximately 10 weeks of age, however, in females this does not occur to around 30 weeks of age, coinciding with the commencement of ovarian

cyclicity (371). Puberty is timed in part by growth, and in females, external cues such as photoperiod (369, 372). The onset of puberty is defined by the hypothalamic decrease in sensitivity to the negative effects of gonadal estradiol, stimulating an increased GnRH pulse frequency and subsequently increased tonic LH secretion from the pituitary (371). It is the release of the pre-pubertal gonadotrophin suppression that initiates sexual maturation and reproductive competence (369). Studies of prenatal androgenisation in females have also identified the role that testosterone plays in programming the timing of puberty onset. These confirm that exposure of a female ovine fetus to androgens earlier, from d30-90 of gestation, led to a masculinised external phenotype (373, 374), whereas exposure later, from d89-135, did not (374). However, in addition to studies investigating the effects of prenatal androgens on external and behavioural masculinisation, studies by Wood *et al*. and Herbosa and Foster, showed that the timing of puberty (increase in tonic LH) and defeminisation of the LH surge is also programmed before birth. *In utero* androgen exposure led to the advancement of puberty in females, illustrated by an early increase in LH secretion (between 16-20 weeks of age) independent of photoperiod, with the critical gestational period for this programming determined to be earlier (d30-90), rather than later (372-374).

Herbosa and Foster also demonstrated that in early (d30-76) treated female sheep, the estradiol positive feedback pathway was diminished. These animals did not display an LH surge following stimulation with a large dose of estradiol indicating that this ovarian-pituitary feedback system was lacking. In the later treated (d89-135) group, the LH response to estradiol did occur, however, the timing of the surge was delayed (374). Interestingly, a study by Wood *et al.* showed that d30-51 and d65-86 prenatal testosterone treatment regimes both led to the differential effects in external masculinisation as described above and also to mutual advancement of puberty (375). Treatment from d30-86 resulted in defeminisation of the LH surge, however, this effect was absent in animals exposed to androgens during the two shorter periods (d30-51 and d65-86), who responded to estradiol, albeit significantly later than controls (375). These findings suggest that the length of prenatal androgenisation, as well as the timing, may be of importance in programming adult neuroendocrine function. Additionally, the dose of testosterone administered can affect the overall phenotype. Kosut *et al.* identified a dose-response effect for the consequential external masculinisation and timing of puberty effects of prenatal androgens, however, the estradiol positive feedback system was impaired at all doses (376), suggesting that this pathway is particularly vulnerable to an altered prenatal steroid environment.

The differential effects of androgens and estrogens have since been implicated in the programming of these reproductive traits. Since testosterone is readily converted into estrogen by aromatase, of which there are large amounts present in the placenta, the programming effects of androgens may have androgenic and/or estrogenic origins. Increased fetal serum estradiol was in fact recently demonstrated following prenatal testosterone administration in sheep from d30-90 of pregnancy (377). Non-aromatisable DHT has been commonly used in parallel with an aromatisable androgen, such as testosterone propionate (TP) or testosterone cypionate, to delineate between the effects of androgens and estrogens. The prenatal effects of androgens in controlling positive and negative feedback have been investigated in this way. Masek *et al.* treated pregnant ewes with either DHT or testosterone from d30-90 of gestation. They found that both DHT and testosterone treatment resulted in the advancement of puberty in female offspring, however only TP treatment diminished the LH surge response to estradiol (353). These results imply androgenic programming of the hypothalamus but an estrogenic programming effect regulating estradiol positive feedback to the pituitary.

These early studies describe ovine prenatal androgenisation experiments where the established method for monitoring hypothalamic-pituitary function is the ovariectomised model with addition of exogenous estradiol back into the animal to mimic ovarian production of this steroid (OVX + E_2). This method avoids the effects of other ovarian factors on this system that might distort the findings related to estrogen signalling as well as standardising the amount of estradiol that each animal is exposed to. While this provides a more controlled steroid environment, the endogenous hormonal profile from ovary-intact animals is important to study to identify the true physiological impact of prenatal androgens. For instance, Birch *et al.* found that while exposing fetuses to testosterone from d30-90 or d60-90 resulted in advancement of the initiation of tonic LH increase in gonadectomised animals, in ovary-intact animals ovarian cycling, as indicated by raised progesterone secretion, begun at the same time as control animals at around 30 weeks of age (355). Sharma *et al*. similarly did not find any advancement in the timing of puberty in prenatally d30-90 or d60-90 androgenised ovary-intact female sheep assessed by progestogenic cycling, although the timing of increased LH secretion was advanced. However, these animals did display a diminished estradiol positive feedback response (378) as described in the OVX + E_2 model (378). Another study carried out by Sarma *et al*., found neither advancement nor

normal timing of puberty, but rather a 4 week delay in the commencement of cyclicity in ovary-intact d30-90 prenatally androgenised ewes (352).

The differential effects of androgens and estrogens on the reproductive outcome in ovary-intact sheep have been studied. Veiga Lopez *et al.* determined the differential effects of DHT and testosterone on positive and negative estradiol feedback (354). During the pre-pubertal stage (5 months of age) a GnRH antagonist was initially given to dampen endogenous ovarian estradiol. A small dose of exogenous estradiol was then administered to animals to reflect luteal phase estradiol levels. In this study, unlike other ovary-intact investigations (355, 378), an increase in tonic LH secretion was found to occur at a younger age in both DHT and testosterone prenatally treated female offspring (354). Administering a large dose of estradiol, equivalent to that found in the follicular phase, resulted in a normal LH surge in both control and DHT but not testosterone treated pre-pubertal animals (354). These results agree with the findings of earlier studies in the OVX + E_2 model (353) that estradiol positive and negative feedback are differentially regulated by androgens and estrogens.

Veiga-Lopez and colleagues have also demonstrated the differential effects of prenatal androgenisation on hormonal and ovulatory dynamics during estrus. They have shown that during the first breeding season it is testosterone and not DHT treatment, from d30-90 of gestation, that leads to increased peri-ovulatory estradiol concentrations, as well as a delayed LH surge in response to estradiol, which is additionally dampened in comparison with controls (354). The increased level of estradiol secretion during the follicular phase is a likely consequence of an increased number of antral follicles pertaining to the polyfollicular phenotype, that are observed in testosterone but not DHT-exposed animals (301). These effects appear to have an estrogenic mode of programming that is distinct from other reproductive traits of prenatal androgenisation. For example, hypergonadotrophism is observed in female offspring from sheep prenatally exposed to both testosterone and DHT (379), suggesting an androgenic programming effect.

The timing of *in utero* androgenisation can also affect the hormonal dynamics in the adult. Studies have shown that prenatal androgen treatment from gestational d30-90 but not d60-90 resulted in the dampening of the LH surge in response to increased estradiol (378, 379). The reduced LH surge observed in these animals does not appear to be linked to the absolute amount of estradiol secreted by the ovary since peri-ovulatory estradiol secretion is increased

(354, 380) and administering high levels of exogenous estradiol to these animals pre-pubertally also results in a diminished LH surge (353, 378). This suggests that there may be impairment at the level of the hypothalamus and/or pituitary gland. It is possible that the pituitary receptivity to estrogens may be altered or desensitised (379) or that the pathophysiological increase in LH pulse frequency and amplitude during the follicular phase (352) may account for more depleted pituitary gonadotroph stores. Prenatal testosterone treatment from d60-90 does not affect cycle regularity or luteal function, evidenced by normal preovulatory estradiol, and unaltered luteal progesterone secretion or length, in the first or second breeding seasons (299, 355). Conversely, in d30-90 treated offspring, while regular cycles present in the first breeding season, animals become anovulatory by the following season (355). Prenatal androgens from d60-90 does however lead to an increase in the LH pulse frequency during the follicular phase and still affects the timing of the LH surge, which is delayed following a peak in estradiol, although the amplitude of the LH surge is unaffected (299).

The LH hypersecretion and increased pulse frequency and amplitude that is observed in the prenatally androgenised ewe (299, 352) also suggests a defect in estradiol negative feedback. In the pre-pubertal d30-90 androgenised sheep, there is an increase in LH pulse frequency and amplitude and a greater LH to FSH ratio (352). This latter effect is thought to reflect the increase in LH secretion rather than a decrease in FSH secretion, since administration of a GnRH antagonist ablated LH secretion but only had a small dampening effect on FSH (352). Furthermore, GnRH bolus stimulation leads to an increased response in LH but not FSH in both d30-90 testosterone and DHT exposed animals (379). This indicates that the reduction in estradiol negative feedback is mediated by an increase in sensitivity to GnRH, that this is an androgenic programming effect, and also provides evidence for the differential control of LH and FSH secretion in these animals (379). Given that these hormones are secreted from the same gonadotroph and that estradiol negative feedback is known to regulate both LH and FSH secretion (381) this is unexpected. It is possible that other modulators of FSH secretion may play a role, of which activin, inhibin and follistatin have been proposed (382). One piece of evidence for this is the differential effect of estradiol stimulated LH and FSH release from the ovine pituitary (383). Baratta *et al.* demonstrated that stimulating ovine pituitary cells with estradiol *in vitro* resulted in diminished FSH but augmented LH secretion. *FSHB* and *INHBB* subunit mRNA was also reduced, suggesting a role for activin B in activating

FSH secretion. Indeed, incubating pituitary cells with an activin B neutralising antibody reduced FSH secretion but did not affect LH secretion (383).

Mannikam *et al.* published extensive adult pituitary gene analyses in d30-90 testosterone treated female offspring which did not, however, reveal alterations in the expression of *INHBB*, *INHA*, *FST* or *β-Glycan* subunits, although there was a treatment related up-regulation of an *ACVR1* (379). Interestingly, *GnRHR*, *ERα* and *ERβ* gene expression were unaffected as were *LHβ* and *FSHβ* subunits, however, the common α gonadotrophin subunit was up-regulated by prenatal testosterone exposure (379). Since *ACVR1* was up-regulated but there was no affect on *LHβ* expression, these authors suggest that the increased LH secretion observed in these animals may occur via post-translational modifications of the LH*β* subunit, alterations to GnRHR or ER degradation (379).

In the ovary, the expression of activin and inhibin subunit and follistatin mRNA have also been examined in prenatally androgenised offspring (301). West *et al*. measured follicular mRNA expression by *in situ* hybridisation in ovaries from 5 week old animals that were exposed to testosterone or DHT *in utero* from d30-90. The study found a significant increase in the number of follicles expressing *FST* and a trend for decreased *INHBB* in testosterone and DHT exposed animals, suggesting that intra-ovarian activin levels could be diminished through an androgenic programming effect (301). The authors hypothesise that a reduction in activin availability could contribute to the selection defects (112) and follicle growth arrest (113) that are present in polycystic ovaries in women (125-128). Indeed, West *et al.* demonstrate increased antral follicle number in prenatally androgenised lamb ovaries. However, the fact that this phenotype is observed in the testosterone and not DHT exposed animals, suggests an estrogenic course of action, and therefore does question the role of activins at this stage given that the authors found that altered *FST* and *INHBB* expression followed androgenic programming (testosterone and DHT) (301). Furthermore, the serum levels of gonadotrophins were not altered by 5 weeks in these animals implying that potential altered ovarian activin levels were not having an endocrine effect on FSH secretion, thereby not affecting growth of follicles in the FSH-dependant stage (301). This suggests that the polyfollicular phenotype and altered activin/follistatin mRNA observed in these lambs may not be causally associated.

1.5 The adrenal gland and PCOS

Like the HPO axis which regulates ovarian function, the HPA axis modulates adrenal function and controls the body's response to stress, primarily mediated through the production of cortisol. This circuitry is regulated and controlled through feedback loops so that increasing levels of cortisol negatively feedback to the pituitary to dampen ACTH secretion (384). The HPA axis maintains physiological homeostasis and functions in a circadian pulsatile manner so that cortisol production during the day is timed (385). In the event of physiological stress the HPA axis is activated to produce a surge in cortisol. Such stressors that are known to activate the HPA axis include physical stress such as hypoglycaemia (386), undernutrition (387), intense exercise (388), and surgery (389), as well as emotional stress (390). The main driver of adrenal steroidogenesis is ACTH which is released from the anterior pituitary (384). Corticotrophs within the pituitary secrete ACTH upon stimulation by corticotrophin releasing hormone, secreted by the hypothalamus (384). ACTH regulates adrenal steroidogenesis through a number of pathways, including the uptake of lipids from plasma into adrenocorticotroph cells and the formation of cholesterol esters (32, 391). Furthermore, ACTH stimulates StAR expression, thus driving the transport of cholesterol across the mitochondrial membrane (392).

The adrenal glands are anatomically situated above the kidneys in the retroperitoneum, and are also responsible for the biosynthesis of mineralocorticoids, which are important for kidney function, and adrenal androgens (31). The adrenal glands are composed of distinct tissue regions that each has a specific role. The inner medullary region is responsible for the secretion of catecholamines, adrenaline and noradrenaline, which are required for the body's 'fight or flight' response. The outer cortical region or 'cortex' produces the corticosteroids. The cortex itself is split into three zones that contain specific cell types responsible for the secretion of hormones. These zones, from the inner to outer cortex, include the zona reticularis, the zona fasciculatis and the zona glomerulosa (31).

1.5.1 Steroidogenesis in the adrenal gland

The adrenal is a major source of hormones and each cortical zone contains the specific enzymes required for conversion of cholesterol precursors into distinct groups of hormones (Fig. 1.9). Within the zona reticularis androgens are synthesised including DHEA, DHEA sulphate (DHEAS), androstenedione and testosterone (384, 393). The glucocorticoids, cortisol and corticosterone, are produced in the zona fasciculata (31, 384). The zona glomerulosa produces the mineralocorticoid, aldosterone (384). For the synthesis of glucocorticoids additional enzymes are involved including the P450 enzymes CYP21 and CYP11B1. The conversion of the androgen DHEA into DHEAS is catalysed by DHEA sulfotransferase 2A1 (SULT2A1). The role of CYP11A, 3β-HSD, CYP17 and 17β-HSD are described in Section 1.1.5. Below is a description of the enzymes involved in adrenal-specific steroidogenesis with focus on the glucocorticoid and androgen pathways.

- **CYP21 (21-hydroxylase)**

In the zona fasciculata, the catalytic conversion of 17α-OH progesterone into deoxycortisol is carried out by CYP21, which is exclusively expressed in the adrenal gland (134). The human fetal adrenal gland also expresses CYP21 which is present from 14 weeks of gestation and beyond (394, 395). In the ovine fetus the presence of CYP21 is well characterised in late gestation due to the role of cortisol in regulating parturition in the sheep (396-398).

- **CYP11B1 (11β-hydroxylase)**

In the final step in the cortisol biosynthesis pathway deoxycortisol is hydroxylated into cortisol by the enzymatic action of CYP11B1, specific to the adrenal cortex (134). Like CYP21, CYP11B1 is present in the developing adrenal gland in humans (395), rodents (399), primates (395) and sheep (400).

- **SULT2A1**

DHEA is sulphated by SULT2A1 to produce DHEAS, which is the most highly produced adrenal hormone (401). SULT2A1 is exclusively present in the adrenocortical cells of the zona reticularis (401)

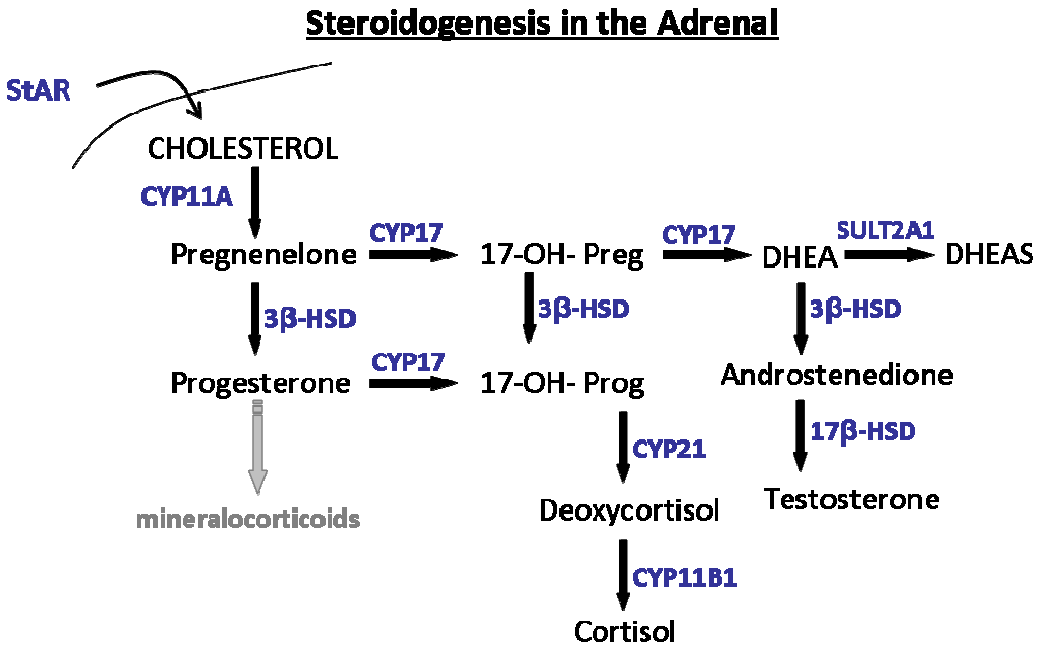

Figure 1.9 The steroidogenic pathway in the adrenal gland. Precursors and products of the steroidogenic pathway are represented in black and the enzymes required for each bio-synthesis step are indicated in blue. Pathways in grey are alternative steroidogenic pathways.

1.5.2 Adrenal androgen production and PCOS

The adrenal gland is a significant contributor to circulating androgens in women. While DHEAS is exclusively produced by the adrenal, the adrenal contribution to DHEA, androstenedione and testosterone is thought to be approximately 50%, 50% and 25%, respectively, with the remainder produced by the ovary or by peripheral conversion (393). Both DHEA and DHEAS are produced by the fetal adrenal gland (402), but their secretion declines after birth until adrenarche at about 6-8 years in the human (403). Adrenarche is a phenomenon that occurs in humans and higher primates (404, 405). At adrenarche the zona reticularis matures and the adrenal produces DHEA and DHEAS, before the onset of puberty and the associated rise in sex steroids (403), without an associated increase in cortisol production (401). During this time there is a reciprocal increase in CYP17 and decrease in 3β-HSD in the zona reticularis to favour androgen production (406, 407). The role of adrenarche in human physiology is however poorly understood (407).

In addition to ovarian hyperandrogenism in women, androgen excess in the form of DHEA and DHEAS secretion is known to occur in some women with PCOS (408-411). Moreover, daughters of women with PCOS have been shown to have an exaggerated adrenarche and produce higher levels of DHEAS than controls (412). The same individuals also had increased serum 17α-OH progesterone after stimulation with ACTH, suggesting increased activity of CYP17 (412). In addition to DHEA, androstenedione levels are also greater in women with PCOS following ACTH stimulation (413, 414). This is suggestive of a heritable programming effect of PCOS that may be induced by maternal hyperandrogenism. Indeed, in the rhesus monkey model of PCOS, basal levels of DHEA were elevated in prenatally androgenised animals compared to controls (297, 415). Zhou *et al.* demonstrated that after stimulation with ACTH, both DHEA and androstenedione were found to be significantly increased in these animals. In control animals DHEA reached peak values 15 min after ACTH stimulation and then declined, however in prenatally androgenised monkeys DHEA levels were still increasing 60 min following stimulation (415). Basally, androstenedione levels were not altered between control and prenatally androgenised group, however 15, 30 and 60 min post-ACTH stimulation, androstenedione was significantly elevated in the treated groups (415). In the control cohort, androstenedione peaked at 15 min, however in the prenatally androgenised monkeys this did not happen until 30 min post-ACTH (415). Interestingly, there were no alterations in the serum levels of 17α-OH progesterone in the rhesus monkey model following adrenal stimulation (415), unlike studies in humans (412-414).

1.5.3 Glucocorticoid production and PCOS

There are numerous downstream roles for the glucocorticoids which are crucial for maintaining homeostasis in a variety of tissues. Cortisol has permissive roles in fat metabolism and hepatic glycogen production from protein. Cortisol also has roles in the central nervous system, cardiovascular control, immune response and inflammation and also acts on muscle, bone and the kidneys (31). Cortisol exerts its effect through binding of the glucocorticoid receptor (GR), which depending on the tissue requirement for cortisol action, will vary in expression. Cortisol is secreted in a diurnal rhythm increasing during the night and low throughout the day (416). This is important for the regulation of metabolic processes that occur during sleep, including gluconeogenesis and lipolysis (417-419). Cortisol negatively regulates insulin action so that it prevents glucose uptake and storage by

peripheral tissues and releases insulin-mediated inhibition of liver glucose production (31). This latter effect on gluconeogenesis occurs through the stimulation of the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK) gene, which is inhibited by insulin (420). The catalytic action of PEPCK is the rate-limiting step of the synthesis of glucose from non-carbohydrate sources such as lactate and its regulation is important in maintaining glucose homeostasis (421, 422). The effect of cortisol on PEPCK expression has also been shown to be long-term in that exposure to high levels of glucocorticoids during late pregnancy in the rat can lead to the permanent up-regulation of hepatic *PEPCK* and *GR* gene expression, culminating in hyperglycaemia (423). Cortisol also increases appetite and therefore caloric intake, and can have a direct effect on fat, stimulating lipolysis and inducing differentiation of adipocytes. Cortisol excess can therefore lead to obesity which is characterised by central fat distribution, and is a distinct symptom of disorders of cortisol production such as Cushing's syndrome (31).

Given the wide ranging and complex roles of cortisol, glucocorticoid action in peripheral tissues must be carefully regulated. Tissues can control the amount of cortisol that can act locally by increasing or decreasing the levels of the inactive metabolite of cortisol, cortisone. This process is regulated by the action of the hydroxysteroid dehydrogenase enzymes, type 1 and type 2 11β-HSD, which interconvert active inactive forms of cortisol (31). Type 1 11β-HSD catalytically converts cortisone into cortisol in cortisol target tissues such as muscle, liver, fat and bone and can act bi-directionally (424). Type 2 11β-HSD is responsible for the inactivation of cortisol by conversion into cortisone. This reaction is important in tissues that are targets of the mineralocorticoids such as the kidney. Glucocorticoids can bind to the mineralocorticoid receptor with a higher affinity than the mineralocorticoid aldosterone, and thus these hormones compete for binding sites. The inactivation of cortisol by type 2 11β-HSD is therefore a crucial requirement for mineralocorticoid signalling in this tissue (31, 424). Cortisol can also negatively regulate its own expression by directly down-regulating GR, which also leads to a reduction in type 1 11β-HSD expression, and therefore cortisol levels decline (31). The placenta characteristically also expresses type 2 11β-HSD, to protect the fetus from elevated maternal glucocorticoids, which might adversely effect the fetus (425).

Like increased adrenal androgen output, studies have revealed that the secretion of glucocorticoids can also be dysregulated in PCOS. Women with PCOS appear to have an

enhanced HPA activation to stress, demonstrated by increased levels of ACTH and cortisol output compared to controls in response to a stress stimulus (426). Similar effects have been found in the Rhesus monkey model of PCOS, in which prenatal androgenisation programmed the increased secretion of corticosterone upon adrenal stimulation with ACTH, although cortisol was unchanged (415). In control animals corticosterone peaked 15 min post-ACTH; however, levels were still increasing in the androgenised animals after 60 min. Also, treatment with the synthetic glucocorticoid dexamethasone, to suppress adrenal steroidogenesis, resulted in a decrease in corticosterone levels in control, but not in the PCOS-model monkeys (415). While not published it was noted in a review by Dumesic *et al.* that basal and ACTH-stimulated levels of cortisol were not altered in the prenatally androgenised sheep PCOS model (427).

1.6 The role of obesity and metabolic dysregulation in PCOS

Obesity and the associated metabolic disorders including type II diabetes have been steadily increasing in Western populations for the last few decades and are now present in epidemic proportions (428, 429). The relatively recent advances in technology and changes in lifestyles have resulted in a sudden shift in daily calorie consumption and a parallel increase in sedentary behaviour (428, 430), which are now becoming more prevalent in adolescents and young adults (431). The negative effects of these abrupt alterations in the external environment are clear, with central fat accumulation linked to various illnesses including cardiovascular disease, hypertension, metabolic syndrome and reproductive dysfunction (430, 432). It is unsurprising therefore that central adiposity is associated with PCOS (166). Increased central obesity leads to insulin resistance, and as food is consumed more and more insulin is required to store glucose as glycogen in the peripheral tissues. A hyperinsulinaemic state evolves to compensate for the increased resistance to insulin in tissues such as muscle and fat in which the uptake of glucose occurs, and to the liver in which glucose release is suppressed (433). Over time this physiological intervention is no longer adequate and glucose homeostasis is inappropriately regulated resulting in hyperglycaemia and the development of type II diabetes mellitus (434).

Insulin acts on peripheral tissues by binding to the to the insulin receptor, a heterotetramer that is composed of two extracellular α-subunits and two membrane spanning β-subunits linked by disulphide bonds (200). Binding of the ligand to the α-subunits results in phosphorylation of the tyrosine kinase of the intracellular β-subunits. This leads to the phosphorylation and activation of downstream signalling cascades, resulting in the recruitment of the insulin receptor substrates which activate either the phosphoinositide 3′ kinase intracellular pathway, involved in driving glucose transport, or the mitogen activated protein kinase pathway, that promotes the mitogenic effects of insulin (433). Perturbation in the expression of some of these pathway constituents in both adipocytes and muscle has been linked with insulin resistance and type II diabetes (435-437). Furthermore, intrinsic abnormalities have been documented in insulin signalling in PCOS. Post-receptor defects have been identified in fat and muscle of women with PCOS (438-440). In cultures of skin fibroblasts from women with PCOS, a defect in insulin receptor signalling was identified where basal activation of the insulin receptor was increased, and hence insulin-stimulation had little effect on receptor auto-phosphorylation compared to controls (441). It has also been shown that the defects affecting insulin signalling in skin fibroblasts are selective so that it is the metabolic pathway and not the mitogenic pathway that is affected (442). In skeletal muscle from women with PCOS, however, there are indications that the mitogenic downstream effects of insulin are altered (443, 444).

There is a positive correlation between increasing central adiposity and insulin, glucose, triglyceride and cholesterol measurements and the insulin resistance index (445). Increased central fat in women is also associated with elevated free total testosterone indexes which are directly regulated by the presence of sex hormone binging globulin (SHBG) which binds androgens, sequestering their activity. SHBG is decreased in these individuals and the subsequent rise in unbound testosterone is associated with male-like fat distribution (430). Approximately 40% of obese PCOS women are insulin resistant (167, 168, 446) which is significantly more prevalent than the levels found in ethnicity, age and weight matched control populations (447, 448). Moreover, women with PCOS are more likely to develop type II diabetes than weight matched controls (449, 450). While central fat mass, insulin resistance and hyperandrogenism are correlated with PCOS (451), not all women with PCOS are obese and a significant number have normal BMIs. It appears that while central obesity and elevated androgens in PCOS are associated with insulin resistance, obesity may exert an additive secondary role to the overall phenotype of insulin hypersecretion, rather than a

causative one (430). Dunaif *et al.* demonstrated that insulin-stimulated glucose utilisation was perturbed in both lean and obese PCOS women, with both subsets experiencing reduced insulin sensitivity compared to controls, however these effects were significantly worse in the presence of obesity (167, 168, 446). These studies indicate that some degree of hyperinsulinaemia may be common to all women with PCOS and it is not entirely due to decreased insulin clearance. This may reflect pancreatic β-cell dysfunction, and that only obese women with PCOS are likely to become glucose intolerant (434, 452).

The hyperandrogenic state present in PCOS combined with hyperinsulinaemia and central obesity, form a vicious circle of positive feedback in which one feature augments the next. There is evidence that androgens can directly increase adipocyte number in cultured female rat adipose tissue (453) and decrease hepatic insulin receptor numbers (454), which could modulate insulin-mediated hepatic glucose suppression. There is also some evidence for the development of mild insulin resistance through exposure to androgens (455, 456), however, treatment of women with androgen antagonists had variable outcomes (200, 457-459). What is known is that hyperinsulinaemia in PCOS can augment androgen production by stimulating ovarian thecal cell androgen production (434, 460, 461). One of the mechanisms that has been proposed to mediate this is the insulin induced increase in thecal 17α-hydroxylase activity (205). Women with PCOS who had been treated with metformin demonstrated a reduction in the levels of 17α-OH progesterone after LH stimulation, and a concomitant decrease in serum androgen levels, compared to women with PCOS who had not undergone the insulin sensitising treatment (205). Furthermore, *in vitro* treatment of cultured porcine thecal cells with insulin in the presence of LH has been demonstrated to lead to the up-regulation of *StAR* and *CYP17* transcripts (462).

Insulin and the insulin receptor are highly homologous to IGF1 and the IGF1R, respectively (463), and insulin and IGF1 can bind to both receptors albeit with less affinity than the native ligand (464). IGF1, IGF1R and insulin receptor genes are expressed in the human ovary (60), and while there is some insulin present in follicular fluid (465) this is thought to be through perfusion from the systemic circulation. In PCOS, the increased concentration of peripheral insulin is proposed to lead to increased stimulation of the ovarian IGF1R and lead to increased steroidogenesis (434). However, it has also been shown that in cultured granulosa cells the inhibition of the insulin receptor but not the IGF1R had a detrimental effect on estrogen and progesterone synthesis (342) suggesting that the insulin receptor is required in

these cells. Likewise in human thecal cell cultures, while both insulin and IGF1 stimulated androgen production (461, 466-469), only inhibition of insulin receptor signalling prevented the increase in androgen biosynthesis (461, 469).

The selective perturbations of insulin signalling pathways leading to metabolic dysregulation in peripheral tissues and insulin resistance may not affect ovarian insulin signalling required for steroidogenesis. Therefore insulin is capable of stimulating androgen production in the ovaries of women that are insulin resistant (468). Indeed studies have shown that in cultures of human PCOS granulosa cells the metabolic but not the mitogenic/steroidogenic actions of insulin are defective (347, 470). Outside the ovary, insulin has other actions to enhance androgen production. Insulin can act directly on the liver to suppress the hepatic production of IGFBPs (471, 472), thereby further augmenting free IGF1 levels in PCOS. In addition, insulin can negatively regulate the production of SHBG by the liver in women with PCOS (472, 473), hence further increasing the bioavailability of androgens. Additional evidence for the role of insulin in enhancing circulating androgen levels are the findings that treating women with PCOS with insulin sensitising drugs such as metformin or troglitazone (204, 213), or with drugs that inhibit insulin secretion including diazoxide or somatostatin (474, 475), considerably lowers free androgen and increases SHBG levels.

1.6.1 Metabolic dysregulation in the prenatally androgenised animal model of PCOS

The metabolic features of early androgen exposure as well as the reproductive characteristics are clear in models of PCOS in rodents (365, 476), non-human primates (298, 477) and sheep (478, 479). Pre-pubertal administration of DHT to female rats leads to increased body weight and fat, and enlarged adipocytes, as well as raised circulating leptin and insulin resistance (365). Other studies have shown a re-distribution of fat in female mice exposed to androgens peri-natally so that there is an increase in mesenteric (visceral fat) and also an increase in lean muscle mass (480). Assessment of prenatally androgenised female mice revealed hyperglycaemia and a reduced glucose tolerance that was caused by impaired pancreatic islet function (476). *In vitro* treatment of pancreatic islet cells with androgens also resulted in diminished response to glucose stimulation implying that both programming by androgens and elevated endogenous androgens in the adult can contribute to the metabolic phenotype (476).

In the prenatally androgenised Rhesus monkey model, differential metabolic defects were detected in adult animals as a result of early and late gestational androgen exposure. In early treated offspring pancreatic β-cell function was impaired with a decrease in acute pancreatic responsiveness to glucose, however, in late treated offspring insulin sensitivity was decreased and this was correlated with increasing BMI (298). Assessment of the fat distribution in adult animals that were exposed to androgens early in gestation revealed that total abdominal fat and visceral fat were significantly increased compared to controls at the expense of other fat depots, indicating a re-distribution of fat reserves (477). In late TP treated Rhesus monkeys however, there was an overall increase in the percentage of total body fat and central adiposity compared to early treated and control animals (481). In the ovine model of PCOS, in which the pregnant ewe was administered androgens from d30-90 of gestation, increased insulin concentrations and a reduced sensitivity to insulin were reported in 5 week old offspring (478). The same metabolic defects were observed in 11 week old lambs that were prenatally treated with TP or DHT from d30-90, indicating that these traits are programmed by androgenic actions (479). Furthermore, the time window in which this programming occurs was narrowed down to mid-gestation, since androgenisation from d60-90 resulted in a similar phenotype to the d30-90 offspring (479). Glucose clearance was not however altered in the ovine PCOS model following glucose tolerance testing, implying that instead of a pancreatic defect in these animals, hyper-insulinaemia occurred as a compensatory mechanism to insulin resistance in peripheral tissue (479).

In addition to the early programming of metabolic dysfunction in the PCOS model, the direct effect of obesity was examined in the prenatally androgenised sheep (479, 482). Offspring that had been prenatally exposed to TP from d30-90 were over-fed from 14 weeks of age until they were 25% heavier than controls. Ovarian cycling defects and perturbations in hormonal dynamics were most profound in obese TP-treated offspring compared to non-obese TP-exposed animals. Obesity in control animals also led to mild reproductive anomalies compared to the TP-treated animals (482). At 20 weeks of age insulin sensitivity was severely impaired in the obese TP-exposed animals, compared to the non-obese TP treated ewes, and obese controls also had a mild phenotype that was not as pronounced as the non-obese TP-exposed animals (479). Therefore while prenatal androgens can programme a pre-disposition to be insulin insensitive, the external environment can significantly augment this phenotype. This draws parallels with the impact of obesity in women with PCOS. Furthermore, the clinical studies that show improvement of the metabolic and reproductive

aspects of PCOS through treatment with insulin sensitisers (204, 213) have been replicated in the sheep model. A sub-group of adult sheep that were exposed to TP from d30-90 of gestation were treated with the insulin sensitiser rosiglitazone for 8 months commencing after the first breeding season (483). This resulted in reduced insulin concentrations and improved insulin sensitivity, compared to TP-treated animals without insulin sensitising treatment. This treatment also resulted in a much higher proportion of ewes that were cycling in the second breeding season in comparison to no intervention (483). Similar results were described in the prenatally androgenised Rhesus monkey model in which treatment with pioglitazone, an insulin sensitiser, normalised disrupted ovarian cycling in two thirds of animals (484).

1.7 The objectives of this thesis

As discussed, PCOS is a complex disorder encompassing a variety of features that present with differing permutations and severity from patient to patient. The advent of the prenatally androgenised animal model has allowed for research into the origins of this disorder, which appear to be centred on the fetal programming effect of steroid hormones including androgens and estrogens. Studies that have administered alternative androgens to the mother, such as DHT that cannot be aromatised into estrogens, have aided the current understanding of the differential effects that the steroid hormones can exert. In this thesis, a novel model of prenatal androgenisation is introduced, with the objective of improving or adding to the present knowledge of the origins of PCOS. This intervention involves the delivery of androgens directly to the ovine fetus during mid-gestation and by-passes the maternal circulation and thus potential placental steroid modification effects (Chapter 3). Over development and in adult life, the reproductive, endocrine and metabolic aspects that are common to PCOS, and present in other animal models, are assessed and compared to an established mid-gestational maternal model of prenatal androgenisation.

In addition to assessing the adult hormonal (Chapter 6) and ovarian (Chapter 7) dynamics that are central to PCOS, a number of additional, and novel, pathways or features are also investigated throughout this thesis. These include the examination of the role of genes and proteins that have developmental roles such as cell differentiation, proliferation and apoptosis, including the inhibitors of differentiation (Ids) and the Roundabout (ROBO) and SLIT family, in the fetal ovary (Chapter 4). This characterisation of the Ids for the first time in the mammalian ovary prompted a thorough investigation of Id protein localisation and gene regulation in the normal adult ovary (Chapter 5) and the ovary after prenatal androgenisation (Chapter 7). In addition, this ovine model of PCOS is used to investigate novel alterations in adult metabolic tissues, including adrenal function (Chapter 8), the development of fatty liver, and the expression of the gluconeogenic enzyme PEPCK and the growth factor, IGF1, in adult livers (Chapter 9).

The work presented in this thesis reveals the effects of prenatal androgenisation during development, which perturbs ovarian, adrenal and liver gene expression. In the young adult animal, a thorough endocrine and ovarian assessment demonstrates that mid-gestation

androgenisation of the ovine fetus does not result in the hormonal or ovarian morphological features typical of adult PCOS. This provided a unique model in which to investigate intrinsic programming alterations in the ovary in the absence of high levels of LH and androgens. Major findings that are discussed in this thesis include an enhanced drive for ovarian and adrenal androgen production, and early indications of metabolic and hepatic dysfunction, in adult prenatally androgenised offspring. In addition, a substantial part of this work involved the characterisation of the direct fetally androgenised cohort which revealed a separate but common phenotype, occurring in both control and androgenised animals. This surprising finding highlighted two important points that suggest that: A) a combination of pathways may programme the adult features of PCOS that could occur independently or be additive, and B) a relatively uninvasive fetal and/or maternal procedure is sufficient to induce effects independently of androgen manipulation.

Chapter 2

General Materials and Methods

2.1 Introduction

This chapter details the general materials and methods that were employed in this thesis. Any work that was carried out in conjunction with others is duly acknowledged in the chapters in which the specific work was presented. All reagents and chemicals listed in this thesis were purchased from Sigma-Aldrich (Gillingham, Dorset, UK), unless otherwise stated.

2.2 Animal husbandry

A flock of Scottish Greyface ewes were purchased for these studies and housed and cared for at the Marshall Building, Roslin, Midlothian under the regulations authorised by UK Home Office Project Licence Number 60/3744. The animals included in these studies were bred over two consecutive years from 2007-2009.

2.2.1 Mating and pregnant ewe husbandry

Ewes with good body conditioning scores (2-3) were mated with Texel rams under natural seasonal breeding conditions from November to January. Prior to mating, estrous cycles were aligned in two separate groups that were staggered over two weeks to achieve pregnancy at the same time for the majority of animals within each group. Chronogest CR sponges impregnated with 20mg flugstone acetate (Intervet UK Ltd., Buckinghamshire, UK) were inserted into the vagina for 10-12 days. At sponge withdrawal animals were given 0.5ml prostaglandin estrumate (i.m.; Schering Plough Animal Health, Welwyn Garden City, UK) to terminate the luteal phase. Ewes not carrying a reference paint marker on their hind to indicate ram-ewe contact, were considered not mated, and were returned for mating a second time. Pregnancy was confirmed by ultrasound scan and ewes placed into treatment groups according to the number of fetuses (singleton, twin or triplet pregnancies). Pregnant ewes were housed in groups in spacious enclosures and fed hay *ad libitum*, supplemented with Excel Ewe Nuts (0.5-1.0 kg daily; Carrs Billington, Lancashire, UK) and Crystalyx Extra High Energy Lick (Caltech Solway Mills, Cumbria, UK). To avoid dominant females from over-feeding at the expense of smaller ewes, these animals were housed in separate pens. All ewes were boosted against Clostridial diseases and Pasteurellosis 4-6 weeks before lambing by treatment with Heptavac P Plus (Intervet UK Ltd.).

2.2.2 Pregnant ewe treatment regimes

Treatment regimes began at gestational d60-62 of an average 147 day gestation and are illustrated in Table 2.1. TP (AMS Biotechnology (Europe) Ltd., Abingdon, UK) was dissolved in vegetable oil (Sainsbury's SO organic range) and 5% ethanol to a concentration of 100mg/ml and stored in an incubator at 37°C, to prevent crystal formation, until ready to use. Vehicle controls (C) consisted of vegetable oil and 5% ethanol and was stored in the same way. Maternal injections were delivered (i.m.; 1ml) into the hind region and included ewes carrying singleton, twin and triplet pregnancies of which there was an equal distribution for control or TP groups. Fetal injections were carried out only in singleton or twin pregnancies following anaesthesia of the mother. Ewes were sedated using 10mg Xylazine (i.m.; Rompun; Bayor Plc Animal Health Division. Berkshire, UK), left to settle for 10 min, then given 2.0mg/kg ketamine (i.v.; Keteset; Fort Dodge Animal Health, Southampton, UK). Ewes were additionally administered a post-operative dose of antibiotics (1ml/25kg; i.m.; Streptacare; Animalcare Ltd., York, UK). Under surgically sterile conditions, injections were performed with the guidance of ultrasound using a vaginal probe fitted with a needle guide. A 20G Quincke spinal needle (BD Biosciences, Oxford, UK) was inserted through the uterine wall and into the flank of the fetus. Delivery of fluid into the peritoneal cavity (200µl) was monitored by ultrasound and any anomaly such as leakage into amniotic fluid or uptake into the fetal circulation was recorded. Ultrasound was performed by Dr Colin Duncan and fetal injection carried out by Professor Alan McNeilly or Kirsten Hogg according to the licensing conditions permitted by the UK Home Office.

Gestational Day (d)	Treatment	Conc.	Collection Stage	Offspring Sample Size (n)
Maternal Treatment Regime				
d60/62-102 twice weekly	C	-	Fetal (d70)	F:3 , M:8
			Fetal (d90)	F:6 , M:12
			Lambs (12 weeks)	F:9 , M:5
			Adults (11 months)	F:5
	TP	100mg	Fetal (d70)	F:6 , M:5
			Fetal (d90)	F:8 , M:13
			Lambs (12 weeks)	F:7 , M:11
			Adults (11 months)	F:9
Fetal Treatment Regime				
d60	C	-	Fetal (d70)	F:4 , M:5
	TP	20mg	Fetal (d70)	F:8 , M:3
d62 and d72	C	-	Lambs (12 weeks)	F:6 , M:11
			Adults (11 months)	F:4
	TP	20mg	Lambs (12 weeks)	F:6 , M:10
			Adults (11 months)	F:8
d62 and d82	C	-	Lambs (12 weeks)	M:6
			Adults (11 months)	F:4
	TP	1mg	Lambs (12 weeks)	M:4
			Adults (11 months)	F:5
	TP	4mg	Lambs (12 weeks)	M: 10
			Adults (11 months)	F:5
	TP	20mg	Lambs (12 weeks)	M:2
			Adults (11 months)	F:7

Table 2.1 The pregnant ewe treatment regimes and resulting offspring. Timings of treatment regimes for pregnant ewes and a breakdown of treatment cohorts, listing concentration of TP delivered, age of sacrifice and collection, and number of offspring present in each group. F: female, M: male.

2.2.3 Husbandry and assessment of lamb and young adult offspring

Lambs were suckled or supplemented with a powdered lamb milk substitute (Shepherdess, SCA Mill, North Yorkshire, UK), then weaned gradually onto hay and grass, away from dams, at 12 weeks. Lambs were also vaccinated at 3 and 7 weeks with Heptavac P Plus (Intervet UK Ltd.). Various parameters were assessed in offspring from birth until 11 months of age, to assess the effects of prenatal androgenisation on growth, metabolic homeostasis and adrenal sensitivity. These included glucose tolerance testing (GTT), synacthen testing and serial blood sampling and are described in detail in the specific chapter in which these methods are included.

2.3 Animal sacrifice and collection of specimens

2.3.1 Fetal, lamb and adult animal sacrifice

Pregnant ewes were euthanised at d70 or d90 of pregnancy and their fetus(es) immediately collected, or lambs were born and sacrificed at 3 months or 11 months of age. For 11 month old animals, estrous cycles were synchronised so that animals were sacrificed during estrus. Ewes received two injections of 125mg prostaglandin estrumate (i.m.; Schering Plough Animal Health) 11 days apart to ensure that all cycles were aligned. Euthanasia was carried out in accordance with the regulatory outline of Schedule 1: Appropriate Methods of Humane Killing of the Animals (Scientific Procedures) Act 1986. A single lethal injection of pentobarbitone sodium (150mg/kg; i.v.; Euthetal; Merial Animal Health Ltd., Essex, UK) was administered to the animal.

2.3.2 Tissue and plasma collection

A range of tissues were collected from animals for studies outlined in this thesis, as well as ongoing studies in the Duncan laboratory (Table 2.2). Fetal blood was also collected in heparinised tubes at the time of sacrifice, spun at 3000 rpm at 4°C for 15 min and the plasma stored at -20°C. Weights of various postnatal tissues were recorded immediately following dissection and before excising a small portion for fixing or freezing. These included lamb or adult ovaries, testes, adrenal glands, pituitaries and in the adult only, omental fat. Tissues were either fixed in Bouins solution, to prevent tissue degradation and maintain tissue integrity, or snap frozen in dry ice followed by storage at -80°C, to prevent RNA degradation. In adult cohorts, an additional liver sample was collected and embedded in cassettes containing OCT compound (VWR International, Leicestershire, UK) and snap frozen in dry ice. These samples were stored at -80°C.

Tissue	Fetal (d70/90)	Lamb (12 weeks)	Adult (11 months)	Maternal Tissue*
Adrenal Gland	✓	✓	✓	✓
Fat subcutaneous	✓	✓	✓	✓
Fat visceral	✓	✓	✓	✓
Heart	✓			
Kidney	✓			
Muscle	✓	✓	✓	✓[2]
Liver	✓	✓	✓	
Lung	✓			
Pancreas	✓	✓	✓	
Placenta	✓			
Thymus	✓			
Thyroid Gland	✓	✓	✓	
Spleen	✓			
Testes/Ovaries	✓	✓	✓	✓
Epididymis		✓		
Uterus	✓	✓	✓	✓
Mammary Gland	✓	✓[1]	✓	✓
Scrotum	✓[1]			
Skin	✓[1]			
Pituitary Gland	✓[1]	✓	✓	

Table 2.2 Bank of ovine tissues collected at the time of sacrifice. * Tissue collected from the mothers during fetal collection. [1]Tissue fixed only. [2]Tissue snap frozen only. N.B. Not all of the tissues listed are studied in this thesis.

2.4 Histology

Tissues including the ovary and liver were processed for histological assessment in the studies described in this thesis.

2.4.1 Tissue fixation, processing and sectioning

Tissues were fixed in Bouins solution at the time of collection for 24 h and then transferred to 70% ethanol. Samples were processed in an automated Leica TP1050 tissue processor (Leica Microsystems, Milton Keynes, UK) followed by embedding in heated liquid paraffin wax and cooled into blocks for long term storage at room temperature. Tissues were sectioned at a thickness of 5µm using a Leica RM 2135 microtome and wax ribbons were transferred to a pre-heated water bath (55°C), to float the sections to remove creases in the tissues. Sections were mounted onto charged slides (Superfrost Plus, ThermoScientific, Gerhard Menzel Gmbh, Braunschwein, Germany) and placed in an incubator at 50°C overnight to dry.

2.4.2 Haematoxylin and eosin staining

In order to visualise tissue morphology microscopically, haematoxylin and eosin (H&E) staining was performed. Haematoxylin stains cell nuclei blue/purple and is counterstained with eosin which leaves a pink residue in the cytoplasmic region of cells. This technique allows for the determination of differential tissue patterns, or, the identification of particular cell types such as the granulosa cells of an ovarian follicle. Sections were de-waxed in xylene (VWR International; 2x5 min), re-hydrated through a series of alcohols (100%, 100%, 95%, 70% ethanol and distilled H_2O (dH_20) for 20 sec each) and tissue was stained in haematoxylin for 1 min before rinsing in dH_2O. Tissue was very briefly rinsed in acid alcohol (1% HCl in 70% ethanol) to remove excess traces of purple stain from the glass slide and washed in dH_20. Sections were placed in Scott's tap water (blueing reagent) for 30 sec and rinsed dH_20, before immersion in eosin for 30 sec and a further washing step. The tissue was subsequently dehydrated through a series of alcohols (70%, 80%, 95%, 100%, 100% ethanol for 20 sec each) and cleared in xylene for 5 min, before mounting with a cover slip

(VWR International) using pertex mounting media (Cell Path, Hemel Hempstead, UK). Slides were allowed to dry for a sufficient time in a fume hood before visualisation of tissue using a light microscope.

2.4.3 Immunohistochemistry and immunofluorescence

The immunolocalisation of proteins in tissues can be achieved using immunohistochemistry and immunofluorescence techniques. The former involves visualisation of antigen binding via a colourimetric enzymatic reaction, while the latter consists of detection of a bound fluorescent signal at the protein of interest. The advantage of immunofluorescence is that dual localisation may be carried out so that two different proteins assigned to fluorophores emitting different spectrums of colour can be visualised. In this thesis both types of protein localisation were employed.

2.4.3.1 Immunohistochemistry

This technique utilises the antibody to antigen binding principle that is employed by the immune systems of mammalian species to combat foreign invasion of bacteria or viruses. Primary antibodies recognise a specific antigen epitope and bind strongly to it when they come into contact. In the direct method of immunohistochemistry this is a one step process where the primary antibody is bound directly to a reporter molecule, however, this method can be insensitive due to the low abundance of binding. More common is the indirect method that uses two or even three layers of complexes to considerably increase binding and amplify the signal. Secondary antibodies bind to the bound primary antibody thus adding a second layer that will involve multiple binding to an antigen. They are often chemically linked to biotin or peroxidase, which recruit binding proteins that consist of the tertiary antibody or complex. This complex contains an enzyme such as alkaline phosphatase or horseradish peroxidase (HRP) and binds with a high affinity to the secondary antibody. In the case where a biotinylated secondary antibody is used the ABC tertiary complex is applied which is consists of an avidin-biotin-peroxidase complex. When HRP-conjugated secondary antibodies are employed a tertiary step is applied consisting of addition of a peroxidase anti-peroxidase immunoglobulin (IgG) complex. The final step in the process involves the addition of a compound (substrate) which reacts with the enzyme linked to the tertiary

complex to form a strong colour or stain. A commonly used organic compound for this purpose is 3,3'-diaminobenzidine (DAB), which is oxidised in the presence of peroxidase producing a brown colour.

2.4.3.1.1 Antigen retrieval

It is often necessary to include an antigen retrieval step to the immunohistochemistry protocol. This process, usually by heating but can be chemical, breaks the cross linking of proteins that can be induced by the fixative and thus exposes the antigen epitopes for antibody binding that might otherwise be inaccessible. In these studies cross-links were removed by heating in a citrate based buffer. Following de-waxing and dehydration (described in Section 2.4.2), slides were completely submerged in a pressure cooker containing citrate buffer (0.05M, pH 6.0) and pressure cooked for 5 min before cooling for 20 min. The sections were rinsed in dH_2O and then washed in phosphate buffered saline (PBS; 2x5 min).

2.4.3.1.2 Blocking steps

A caveat of utilising a technique based on a biological binding principle is the occurrence of naturally occurring molecules in tissues and cells that can interfere with exogenously introduced molecules and thereby increase the risk of non-specific binding and false-positives, otherwise known as ‘background staining’. There are a number of steps in an immunohistochemistry protocol to reduce the extent of non-specific binding of endogenous molecules present in the tissue. The first step is blocking of endogenous peroxidase activity that must be carried out when implementing peroxidase-based detection systems. Quenching of peroxidase activity was performed by the addition of 3% hydrogen peroxide (diluted in dH_2O) for 10 min and further PBS washing (2x5 min). In addition to the presence of endogenous peroxidase, endogenous biotin will also be present in some tissues, which may result in non-specific staining when the biotinylated secondary antibody system is employed. To mask these sites an avidin/biotin kit (Vector Laboratories, Peterborough, Cambs, UK) was used when necessary. Avidin was added to the section to bind endogenous biotin for 15 min, slides were then washed in PBS and incubated with biotin for 15 min to saturate any

remaining biotin-binding sites introduced by the exogenous avidin. Slides were subsequently washed in PBS. The final blocking step involves the incubation of tissue with normal serum belonging to the host animal that the secondary antibody was raised in. IgGs present in the serum can therefore bind to non-specific sites present in the tissue so that they will be inaccessible to the secondary antibody. Slides were carefully wiped with tissue to remove excess liquid and placed in a humidity chamber containing damp paper towels. If necessary an area around the tissue was isolated with use of a wax pen (Daido Sangyo Co., Ltd., Tokyo, Japan) to introduce a hydrophobic barrier and retain the solution over the tissue. This was especially useful if the tissue size was small (e.g. fetal ovary) so that it could be easily identified, or, if a high concentration of antibody was required then a smaller volume could be added. The appropriate normal serum was incubated with the tissue, in a closed humidity chamber, for 1 h at room temperature.

2.4.3.1.3 Primary antibodies

Both monoclonal and polyclonal primary antibodies are commonly used in immunohistochemistry. Excess normal serum was removed from the slide and the primary antibody, diluted to the appropriate optimised concentration in normal serum, was added to the tissue overnight at 4°C. Negative controls are detailed fully in each experimental chapter. They consisted of primary antibody omission, the use of non-specific IgGs or, where commercially available, binding proteins for that antibody were purchased and pre-incubated in excess with antibody before applying to tissue.

- **Monoclonal antibodies**

Monoclonal primary antibodies are the most specific type as they exclusively bind to one antigen epitope and are produced from clones of immune cells. In the antigen-immunised mouse, spleen cells are collected and fused with myeloma cells, which introduce immortalisation. These hybrid cells are cultured in a selective medium and the supernatant containing secreted monoclonal IgGs is collected. Specific clones are tested for their binding ability and are then propagated *in vitro* or *in vivo*.

- **Polyclonal antibodies**

Polyclonal primary antibodies are a mixture of IgGs that bind to a selection of epitopes present on a specific antigen. Common host animals include mice, rabbits, goats, chickens and sheep that are immunised against a certain antigen. Serum containing the polyclonal IgG is collected and purified.

2.4.3.1.4 Colourimetric detection, counterstaining and mounting

Following incubation with the primary antibody, sections were washed with PBS containing 1% tween (PBST; 2x5 min) and subsequently incubated with the appropriate species secondary biotinylated antibody diluted 1:500 in normal serum for 1 h at room temperature. Slides were washed in PBST (2x5 min) and the tertiary ABC complex (Vector Laboratories) added for 1 h at room temperature as per the manufacturer's protocol. Colourimetric detection was achieved by the addition of liquid DAB substrate (Dako, Glostrup, Denmark) diluted as directed and incubated with the tissue for approximately 30 sec to 1 min. The enzymatic reaction was stopped when slides were placed in dH_2O. Tissue was counterstained with haematoxylin for 1 min and rinsed in dH_2O. Acid alcohol and Scott's tap water were applied as described in Section 2.4.2 (omitting the eosin step) as were dehydration of tissue and subsequent mounting with cover slips and pertex.

2.4.3.1.5 Light microscopy and imaging

Protein immunolocalisation was visualised using a Standard 20 Zeiss light microscope (Carl Zeiss Ltd., Welwyn Garden City, UK) and images captured using an Olympus Provis BX2 microscope (Olympus America Inc., Center Valley, PA, USA), attached to a Canon E0S 30D Microcam camera (Canon Inc. Headquarters, Tokyo, Japan) using Axiovert v4.8 software (Carl Zeiss Ltd.). Images were exported as Jpg files and scale bars and appropriate text embedded in figures using Photoshop CS (Adobe Systems Inc., Mountain View, CA, USA).

2.4.3.2 Immunofluorescence

Similarly to immunohistochemistry, there are various methods for immunofluorescent antigen detection. The direct method involves the sole addition of a primary antibody conjugated to a fluorophore (a fluorescent dye that emits light at a certain wavelength). The indirect method includes the addition of a secondary antibody which is chemically linked to the fluorescent chromagen rather than the primary antibody. Finally, a more sensitive method of indirect detection can be achieved by the addition of a fluorophore-conjugated tertiary antibody that binds the regular secondary antibody and amplifies the signal to a greater extent. The immunofluorescence protocols in these studies implemented this latter method of antigen detection. Tissue sections are initially treated in the same way as for immunohistochemistry (Section 2.4.3.1) up to and including addition of the secondary antibody, however, the dilution of the primary antibody and the type of secondary antibody used is dependant on the type of tertiary detection system employed.

2.4.3.2.1 Streptavidin detection system

For a fluorescent detection system using a streptavidin tertiary complex conjugated to a fluorophore, the primary antibody concentration remains the same as for immunohistochemistry and the secondary antibody is biotinylated. This is a more economical method and is optimal for good quality, reliable antibodies that do not require use at very high concentrations. Following PBST washing (2x5 min) the tertiary complex, Streptavidin Alexafluor -488 (Invitrogen, Paisley, UK) which emits green light when excited at this wavelength and is visible at a reduced wavelength, was added to the tissue section at 1:200 (diluted in PBS) for 1 h at room temperature. From this point onwards slides were incubated, and all washing steps were performed, in the dark to reduce photo bleaching through natural light exposure. Sections were washed in PBST (2x5 min) and re-blocked with the appropriate normal serum for 30 min before application of the second primary antibody overnight at 4°C. On the third experimental day as on day 2, the second biotinylated antibody was added, followed by addition of the tertiary complex, Streptavidin Alexafluor -546 (Invitrogen), which emits a red colour upon excitation. Following PBST washes (2x5 min) sections were counterstained by incubation with 4',6-diamidino-2-phenylindole (DAPI), a nuclear stain that emits blue light, diluted 1:1000 in PBS for 10 min.

Tissue was rinsed in PBS and mounted with cover slips using PermaFluor aqueous mounting media (Fisher Scientific Ltd., Leicestershire, UK).

2.4.3.2.2 Tyramide enhanced detection system

The tyramide enhanced system of detection is optimal for primary antibodies that bind less well to the intended target as it is possible to dilute them 10-fold of that optimised for immunohistochemistry; however, the reagents are more expensive. Secondary antibodies used in this system are peroxidase-conjugated and applied to slides at 1:200 (diluted in normal serum) for 1 h at room temperature. Slides were washed in PBST (2x5 min) and, in the dark, were incubated with Tyr-Fluorescein (green; PerkinElmer, Cambridge, UK) diluted 1:50, in buffer supplied by the manufacturer, for 10 min at room temperature. Following PBST washes (2x5 min) tissue underwent antigen retrieval by placing slides in pre-heated citrate buffer pH 6.0 (0.05M) and micro-waving on full power for 2.5 min. The slides were cooled for 30 min then re-blocked in normal serum for 30 min and the secondary primary added overnight at 4°C. On the third day the secondary peroxidase-linked antibody was applied, followed by the tertiary complex, a Tyr-Cy3 (red; PerkinElmer), as above. Sections were washed, counterstained and mounted as described in Section 2.4.3.2.1.

The advantage of these systems is that they can also be combined so that primary antibodies raised in the same species may be used together in the same experiment, since the secondary antibodies employed are conjugated to different reporter systems. For example, a goat primary antibody could be detected using an anti-goat biotinylated secondary antibody and the streptavidin tertiary system followed by a second goat primary antibody which could be detected using an anti-goat peroxidase linked secondary antibody and the tyramide tertiary system.

2.4.3.2.3 Fluorescent microscopy and imaging

Images were captured using an LSM 510 Meta confocal microscope and Zen 2008 software (Carl Zeiss Ltd.). Images were exported as Jpg files and assembled using Photoshop CS (Adobe Systems Inc.).

2.5 Radioimmunoassay

Radioimmunoassays (RIAs) were employed as a highly sensitive method for detection of a range of hormones in sheep plasma. The technique utilises antigen-antibody binding as an inverse measure of the quantity of an antigen (hormone) in the sample. An antigen of interest is radioactively labelled, often with the gamma radioisotope Iodine-125 (^{125}I) as iodine atoms readily incorporate into the tyrosine residues of a protein. A known concentration of this radiolabelled antigen (tracer) and an unknown concentration of antigen in serum are combined with a primary antibody specific to the antigen. The antigen competes with the tracer for antibody binding, displacing it, so that as the concentration of the antigen in the serum sample increases, the quantity of bound radioactive tracer decreases. The primary antibody is then precipitated by binding to a secondary antibody and the unbound and bound fractions are separated by washing in a detergent based buffer. A solid pellet is formed by centrifugation, the unbound fraction is discarded and the radioactivity of the bound fraction is measured using a gamma counter. There is an inverse relationship between the number of gamma counts in a sample and the concentration of unlabelled antigen which is determined by the use of a standard binding curve.

2.5.1 Serum extraction

In some assays it is necessary to first extract the serum from the sample to prevent non-specific binding of the antibody to serum binding proteins. When optimising the assay an experiment with and without extraction should be performed initially to determine whether differences in binding occur. Assays in this study that were identified as requiring serum extraction included testosterone, androstenedione and estradiol. Extraction volumes were 100µl serum for testosterone, 150µl serum for androstenedione and 200µl serum for estradiol assays. In large glass Pyrex test tubes, serum was mixed with 2ml diethyl ether (VWR International) in a fume hood and vortexed for 3 min. Tubes were placed in a bath containing dry ice and industrial methylated spirit (VWR International), to freeze the serum into a solid state. The solvent containing the extracted sample was poured off into 12x75mm glass test-tubes (Fisher Scientific Ltd.) and the solvent allowed evaporate overnight. Tubes containing the extract were covered and refrigerated at 4°C for storage of up to two weeks. Prior to assay the extract was re-constituted in phosphate gelatine buffer saline (PGBS) assay buffer

(Table 2.3) 30 min before use. The volume of assay buffer added was 300µl for testosterone and androstenedione assays (if assaying testosterone in male serum samples 500µl should be added) and 200µl for estradiol assays. Since only small amounts of estradiol were detectable in the samples, the extraction was performed in duplicate and the assay carried out directly in the extraction tubes.

PGBS 0.1M pH 7.4	Quantity (g)
$Na_2PO_4.2H_20$	11.46
NaH_2PO_4	2.61
NaCl	9.0
Thiomersal	0.1
Gelatin	1.0
Up to 1l with dH2O: pH with HCl	
Phosphate Buffer 0.05M pH 7.5	Quantity (g)
$Na_2HPO_42H_2O$	66.75
$NaH_2PO_42H_2O$	19.5
Bovine Serum Albumin (BSA)	1.0
Thiomersal	0.1
Up to 1l with dH2O: pH with HCl	
Citrate Buffer 0.1M pH 4.0	Quantity (g)
$C_6H_8O_7$	21.0
Na_2HPO_4	17.0
Thiomersal	0.1
Gelatin	1.0
Up to 1l with dH2O: pH with NaOH	

Table 2.3 RIA assay buffers. Reagents and quantities required to prepare buffers specific to the hormonal assay.

2.5.2 Assay protocol

The steroid assays (testosterone, progesterone, androstenedione, estradiol and cortisol) were performed over two days with the addition of antibodies and tracer on day 1 and separation on day 2. Assays were set up in duplicate including total count (T0), non-specific binding (NSB), zero binding (B0) and high and low quality control (QC) tubes. Standards were set up in order of increasing concentration followed by unknown samples, also in duplicate. Firstly, the required volumes of unknown/standard and assay buffer (Appendix A) were added to the 10x75mm polypropylene test-tubes (Sarstedt Ltd., Leicester, UK). The primary antibody (100µl) and tracer (100µl) diluted in assay buffer to working concentration and required amount of radioactivity (Appendix A) in counts per minute (cpm) respectively, were then added to the tubes. Assay buffer (200µl), primary antibody (100µl) and tracer (100µl) were added to B0 tubes. Assay buffer (300µl) and tracer (100µl) were added to NSB tubes. In progesterone and cortisol assays where the serum sample was not extracted, the same volume of charcoal-stripped sheep plasma was included in the tubes containing standards, NSBs and B0s. Tracer alone (100µl) was added to T0 tubes. Tubes were vortexed, covered and incubated at room temperature for a minimum of 4 hours.

The secondary antibody (100µl), consisting of a combination of donkey anti-rabbit IgG (Diagnostics Scotland; T022; 1:60) and normal rabbit serum (NRS; MP Biomedicals, Illkirch, France; 1:600) diluted in assay buffer, was added to all tubes except T0 tubes. Tubes were vortexed, covered and incubated overnight at 4°C. On day 2, 1ml wash buffer (polyethylene glycol 4%, saline 0.9%, triton 0.2%) was added to each tube apart from T0 tubes, and tubes were centrifuged at 3000 rpm at 4ºC for 30 min. The supernatant was discarded and the tubes were carefully blotted to remove excess liquid before processing the assay on a Wizard 1470 Automatic Gamma Counter (Perkin-Elmer Inc., MA, USA) to give raw γ counts.

For non-steroid assays, including LH and FSH, a four day protocol was implemented where the tracer, secondary antibody and wash solution were added on days 2, 3, and 4, respectively, with overnight incubations at 4°C following each step. For the Multispecies Leptin Assay Kit (Millipore), tracer was added on day 2 and the primary antibody precipitated directly in supplied wash buffer containing goat anti-guinea pig IgG on day 3. In-house buffers, standards, and secondary antibodies were employed for the MAIA

Oestradiol Assay Kit (Inverness Medical UK) and it was possible to dilute the supplied tracer to around 12,000 cpm to extend the use of the kit. Detailed descriptions of the antibody concentration, tracer count, standard concentrations, required buffer and volume of each component, for each assay carried out, are listed in Appendix A.

2.5.3 Assay Analysis

Computational software AssayZap (Biosoft, Cambridge, UK) was used to analyse the raw data (γ counts per min) generated from each bound fraction (pellet). This programme automatically constructs a standard curve from a pre-determined method file and calculates results by interpolating unknown values with those of the standard binding curve. Data were exported to an Excel file for further analysis. Quality controls, included in every assay were used to determine inter- and intra-assay co-efficients of variation (CVs). These were usually <10% and are specified in the relevant chapters.

2.6 Enzyme-linked immunosorbent assay

Similarly to immunohistochemistry and radioimmunoassay, enzyme-linked immunosorbent assays (ELISAs) also work on the antigen-antibody binding principle as a method for the detection and quantification of an unknown quantity of protein in a sample. This thesis utilised a commercially available ELISA kit for the determination of plasma insulin (80-INSOV-E01, Alpco Diagnostics, Salem, NH, USA). This assay incorporated the two-site or 'sandwich' enzyme immunoassay format in which 96-well polystyrene microtiter plates were pre-coated with a specific anti-antigen (insulin) IgG. The sample containing an unknown quantity of insulin was added and bound to the coated well. The anti-insulin conjugate, which is bound directly to the reporter molecule HRP, was subsequently added creating a sandwich of antigen bound between two layers of antibody. The amount of bound antigen was then determined by the addition of 3,3',5,5'-tetramethylbenzidine (TMB) substrate initiating an enzymatic colourimetric reaction. The reaction was terminated by adding an acidic stop solution, resulting in a colour change from blue to yellow, with the intensity of colour directly proportional to the amount of antigen present in the sample. The optical density was immediately read on a spectrophotometer (ThermoMax Microplate Reader; Molecular Devices, CA, USA) at 450nm with a reference wavelength of 650nm to control for background. Concentrations were determined by extrapolation from a standard curve, subtracting any background measured by the addition of a set of blanks, and the results calculated using SoftMax Pro software (Molecular Devices). Low and high concentrated calibrators were also included to determine assay precision within and between assays. The insulin ELISA inter- and intra-assay CVs were <6% and 5%, respectively, and the sensitivity was 5.0pg/ml.

2.7 Western blotting

Western blotting is a method for the qualitative and semi-quantitative detection of a target protein in tissues or cells. This method utilises antigen-antibody interactions to bind to and detect a specific protein in a sample. A known quantity of total protein is separated by molecular weight (Mw) using sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE). The proteins are subsequently transferred to a membrane, which is probed with a specific primary antibody and an HRP-conjugated secondary antibody. Chemiluminescent detection is employed to visualise protein bands: contact with the HRP conjugate and the chemiluminescent reagents results in cleavage and production of a luminescent signal in proportion to the amount of bound antibody. The light emitted from this reaction is visualised by exposure of the immunoblot to photographic film allowing for the detection of individual protein bands on the blot. The use of an internal control concomitantly ensures consistent loading and can be used as a reference for semi-quantification of results.

2.7.1 Protein digestion

To extract protein from tissue, a small representative piece was excised over dry ice to ensure that the material did not thaw and the sample was immediately transferred to a 1.5ml eppendorf containing 300µl lysis buffer consisting of 1% NP-40, 150mM NaCl, 5mM EDTA pH 8.0, 50mM Tris-HCl and proteinase inhibitor (1 tablet per 20ml; Roche Diagnostics GmbH, Mannheim, Germany). Samples were kept on ice for subsequent steps. Tissue was homogenated by sonicating twice for approximately 5 sec each with a cooling period on ice in between to ensure the samples did not get hot and allow for maximal digestion with lysis buffer. Samples were centrifuged at 3000 rpm at 4°C for 5 min to pellet unwanted cellular material and the supernatant transferred to a clean 1.5ml eppendorf.

2.7.2 Protein concentration measurement

Protein concentrations were determined using the Bradford assay (485), which is a routine spectrophotomic method that involves quantification through a colorimetric assay. The technique involves the binding of Coomassie Brilliant Blue dye to proteins which shifts the absorbance from 465nm to 595nm. This change can be monitored so that increased absorbance at 595nm is proportional to the quantity of protein present in the sample. To prepare the Bradford reagent, 100mg of Coomassie Brilliant Blue G-250 was dissolved in 50ml 95% ethanol. To this, 100ml 85% (w/v) phosphoric acid was added and the solution was placed on a stirrer for 30 min. A final volume of 1l was prepared with dH_2O and the solution filtered through Whatman grade 1 filter paper. This solution was stored at room temperature and used for Bradford assays. The Bradford assay was performed with the use of an automated Cobas Fara centrifugal analyser (Roche Diagnostics, Welwyn Garden City, UK) and individual steps were programmed as follows. To cuvettes 256µl of filtered Coomassie Brilliant Blue stock solution was added and incubated at 37°C for 100 sec. After 95 sec an initial absorbance reading at 595nm was taken and 25µl sample, diluted in 50µl dH_2O, was added to each cuvette. This mixture was incubated at 37°C for a further 180 sec and a final absorbance at 595nm read. The concentration of samples was extrapolated from a standard curve constructed from the dilution of BSA diluted in dH_2O, ranging from 0-100mg/l. If samples were too concentrated (>100mg/l) they were diluted with dH_2O to fall within the middle of the standard curve. A QC was included in every assay to ensure assay precision. Inter- and intra assay CVs were <5% and <2%, respectively.

2.7.3 Preparation of gels

Polyacrylamide gels were prepared in advance and could be stored at 4°C for up to 2 days before use. Glass plates (BioRad Laboratories Ltd., Hertfordshire, UK) were thoroughly cleaned and assembled, ensuring no gaps were present around the edges of gel casters. The resolving (separating) and stacking gels were prepared as described in Table 2.4, however, tetramethylethylenediamine (TEMED) and 10% ammonium persulphate (APS) were added only immediately prior to pouring of the individual gel. Resolving gels were poured with approximately 1.5cm remaining at the top of the plates. To speed up polymerisation, a layer of dH_2O was carefully pipetted in this space and the gel set for 30 min. The layer of water was tipped off and remaining liquid removed by blotting with tissue. The stacking gel was

subsequently overlaid onto the resolving gel and a 14-well toothed comb inserted. The gel was set for 30 min, before proceeding to Western blotting or storing at 4°C.

Reagent	Resolving (10%)	Stacking (5%)
Acrylamide	3.33ml	1.3ml
TRIS-HCl	2.5ml (1.5M; pH 8.8)	2.5ml (0.5M; pH 6.8)
dH_2O	4.2ml	6.1ml
SDS (10% w/v)	100µl	100µl
APS (10% w/v)	40µL	40µl
TEMED	20µl	20µl

Table 2.4 Preparation of Western blot gels. Reagents and volumes required to prepare 10% and 5% resolving and stacking gels, respectively, for use in SDS-PAGE.

2.7.4 Western blot protocol

Protein was diluted to 4µg/µl in lysis buffer and 20µg combined with 2X Laemmli buffer consisting of 0.1M Tris-HCl pH 6.8, 20% glycerol, 2% (w/v) SDS, 0.16% (w/v) bromophenol blue and 3% β-mercaptoethanol to give a 10µl final volume (5µl protein and 5µl Laemmli buffer). Protein was denatured at 95°C for 5 min and the sample loaded onto 10% SDS-polyacrylamide gels. A full-range rainbow molecular weight marker (5µl; GE Healthcare, Buckinghamshire, UK) was additionally applied to one well per gel. Gels were submerged in a running buffer consisting of 0.025M Tris, 0.192M glycine and 0.1% SDS and electrophoresis performed in tanks (BioRad Laboratories Ltd.) at 100V at room temperature for approximately 1.5 h, or, until the bromophenol blue dye reached the bottom of the resolving gel. For the transfer, sponges and filter paper cut to size were soaked in cold transfer buffer consisting of 0.025M Tris, 0.192M glycine and 20% methanol and layers of sponge and filter paper (x2) were assembled. The gel was carefully placed on the filter paper and the hydrophobic polyvinylidene difluoride membrane (Amersham Hybond-P, GE Healthcare) placed on top. A further layer of filter paper (x2) and sponge was added and compression from top to bottom applied to ensure air bubbles were removed. The whole assembly was kept wet at all times in transfer buffer to prevent the protein drying out.

Transfer of protein to the membrane was carried out in tanks (BioRad Laboratories Ltd.) filled with transfer buffer at 100V at 4°C for 1.5 h. Membranes were blocked in 5% milk powder and tris buffered saline (TBS; pH 8.0) containing 10mM Tris and 0.15mM NaCl with the addition of 1% tween (TBST), for a minimum of 1 h at room temperature and then overnight at 4°C. Membranes were incubated with primary antibodies against the antigen of interest or an internal control such as α-tubulin or β-actin, diluted in 5% milk/TBST, for 1 h at room temperature. Immunoblots were washed repeatedly with TBST for 30 min and incubated with an anti-rabbit- or anti-mouse HRP-conjugated IgG, respectively (1:2000; Dako), diluted in 5% milk/TBST, for 1 h at room temperature. Following further TBST washing for 30 min, protein bands containing bound antibody were visualised with the addition of enhanced chemiluminescent detection reagents (Amersham ECL Plus Western Blotting Detection System, GE Healthcare) as per the manufacturer's instructions. Immunoblots were immediately exposed onto photographic X-ray film (Fujifilm Super RX, Bedford, UK) and processed using a photographic developer (SRX-101A, Konica Minolta Medical Imaging Inc., NJ, USA). The size of the visualised protein band was confirmed with the position of the molecular weight marker.

2.8 Gene expression analysis

2.8.1 RNA extraction

To study the expression level of a specific transcript in a tissue sample the coding region, or messenger (m)RNA was first extracted and purified from the sample. The tissue type governed the method of RNA extraction depending on the composition of lipid, blood or fibrous material.

2.8.1.1 RNA extraction using the Qiagen RNeasy Mini Kit

The Qiagen RNeasy Mini Kit (Qiagen Ltd., West Sussex, UK) was applied to a range of tissues including; fetal ovary and liver, placenta, fetal and adult adrenal and adult pituitary. Under sterile conditions, over dry ice, a small (~30mg) piece of representative tissue was excised and placed in a 2ml eppendorf containing a metal ball bearing (Qiagen Ltd). To each tube 600µl RNeasy lysis buffer (RLT Buffer)/1% β-mercaptoethanol was added and the tissue lysed using a TissueLyser (Qiagen Ltd.) for 4 min at 25Hz. RLT Buffer contains guanidine-thiocyanate, which denatures proteins, inactivating RNases that could degrade RNA present in the sample. Following homogenisation, the samples were centrifuged for 3 min at 13,000 rpm and the supernatant transferred to a fresh 1.5ml eppendorf. The homogenate was thoroughly mixed with 600µl 70% ethanol and transferred to an RNeasy spin column. Subsequent centrifugation and wash steps were performed as per the manufacturer's instructions. On column DNase digestion was included during the protocol as advised to avoid DNA contamination of the purified RNA. RNase free DNase I (Qiagen Ltd.) was incubated on the column for 15 min at room temperature. At the final step, RNA was eluted with 30µl RNase free water (Qiagen Ltd.) and stored at -80°C.

2.8.1.2 RNA extraction using the Qiagen RNeasy Micro Kit

The Qiagen RNeasy Micro Kit was required for the extraction of RNA from cells where a low yield would be anticipated compared to tissues, and involves the use of a similar protocol as the Qiagen RNeasy Mini Kit. In this thesis this kit was applied to the extraction of RNA from cultured granulosa cells, and thecal cells from individual follicles, and is specifically described in Chapters 5 and 7, respectively.

2.8.1.3 Measurement of RNA concentration

RNA concentration and purity were determined using a NanoDrop 1000 spectrophotometer (Fisher Scientific Ltd.). A 1.5µl volume was pipetted onto the fibre optic cable and the concentration of RNA measured based on the absorbance reading at 260nm. The absorbance ratio at 260nm and 280nm (260/280) was also given and is a measure of RNA purity. This value should be ~2.0, indicating a pure RNA sample without the presence of contaminants such as protein, DNA or extraction reagents.

2.8.2 Complementary DNA synthesis

Complementary (c)DNA must be synthesised from mRNA to provide a template for the DNA replication, exerted by Taq polymerase during the polymerase chain reaction (PCR), and DNA amplification. Complementary DNA is synthesised from mRNA by reverse transcriptase (RT), transcribing single stranded DNA from an RNA template. RNA was diluted to a concentration of 100ng/µl in nuclease free (NF) H_2O and cDNA synthesised from 200ng RNA using the High Capacity cDNA Reverse Transcription Kit (Applied BioSystems, CA, USA). Volumes and concentrations of each component are described in Table 2.5 and prepared in 0.2ml thin-walled PCR tubes (Axygen Inc., distributed by Thistle Scientific Ltd., Glasgow, UK). A sample omitting RT was included as a negative control. Using a thermocycler (G-Storm GS1, GRI Ltd., Essex, UK) samples were incubated at 42°C for 1 h followed by 95°C for 10 min to inactivate the enzyme. The resulting cDNA was stored at -20°C and the quality was determined by PCR (Section 2.8.4) for the house-keeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and visualised on a gel (Section 2.8.4.1) before further use in experiments.

Reagent	Volume (µl)	Final Conc.
RT Buffer	2.0	1X
dNTPs	2.0	1mM
$MgCl_2$	2.4	3mM
Random Hexamers	1.0	0.25µM
RNase Inhibitor	1.0	20U
MultiScribe RT	1.0	2.5U
NF H_2O	8.6	-
RNA (100ng/µl)	2	200ng
Final Volume 20µl		

Table 2.5 The cDNA reagents, volumes and final concentrations required for cDNA synthesis. The High Capacity cDNA Reverse Transcription Kit was purchased from Applied BioSystems. dNTP: deoxyribonucleotide.

2.8.3 Primer design and validation

Primers for PCR and quantitative real-time (qRT)-PCR were designed using Primer3 Input v0.4 software (486). Primers were often designed from the bovine (*Bos taurus*) genome and sometimes from the pig (*Sus scrofus*) genome published on the Ensembl Genome Browser database. Primer regions were selected so that the forward and reverse sequence was intron-exon spanning and the resulting product size would be in the region of 150-250 base pairs (bp) long. Primer sequences were 'BLASTed' using the Basic Local Alignment Search Tool (BLAST) provided by the National Center for Biotechnology Information (NCBI) to assess the likelihood of obtaining other similarly sized products using those primers. Where possible the sequences were BLASTed against the *Bos taurus*, *Sus scrofa* and *Ovies aries* (sheep) genomes, available on NCBI, to gauge the level of species conservation. A high degree of homology was often identified, particularly between the cow and sheep genome. Primers were validated by conventional PCR (Section 2.8.4) to ensure binding to one product at the expected molecular weight. To confirm specificity of the gene product DNA sequencing was additionally performed (Section 2.8.5).

2.8.4 Polymerase chain reaction

PCR is a routine method used in laboratories to qualitatively and quantitatively assess gene expression within tissues or cells. The technique is based on the amplification of a small region of double stranded (ds) DNA to assess whether or not a certain gene is expressed in a tissue and how abundant it is. Forward and reverse primer sequences for a specific gene anneal to the 3' complementary sequence of denatured single-strand cDNA template and cycles of DNA replication ensue. DNA (Taq) polymerase I adds dNTPs to the 3'-hydroxyl group of the extending strand, forming a phoshodiester bond with the 5'-phosphate group of the dNTP, and synthesises DNA in the 5'-3' direction. Since the original DNA template double strand is separated into two single strands and elongation occurs on both strands each cycle exponentially increases the amount of DNA in the reaction. The reagents and volume required for a single PCR reaction are listed in Table 2.6. A reaction omitting cDNA was always included as a negative control. PCR reactions were performed using a thermocycler (G-Storm GS1, GRI Ltd.) with a cycling programme set out in Table 2.7

Reagent	Volume (µl)	Final Conc.
Green GoTaq Flexi Buffer	2.0	1X
dNTPs	0.2	0.2mM
$MgCl_2$	0.6	1.5mM
Forward Primer	1.0	0.5µM
Reverse Primer	1.0	0.5µM
NF H_2O	3.7	-
GoTaq Hot Start Polymerase	0.5	0.25U
cDNA	1.0	-
Final Volume 10µl		

Table 2.6 The PCR reagents, volumes and final concentrations used per PCR reaction. The reagents were purchased from Promega UK Ltd., Southampton, UK.

Step		Temp. (°C)	Time (min:sec)
Enzyme activation		95	05:00
dsDNA denaturing	x35 cycles	72	00:30
Primer annealing		55-60*	00:45
Strand extension		72	00:45
Final extension		72	10:00

Table 2.7 The PCR thermo-cycling conditions. * The annealing temperature was pre-determined during validation to the optimal temperature for binding.

2.8.4.1 Gel electrophoresis

The PCR product was run on a 2% agarose gel to visualise a single band. For a 120ml gel, 2.4g agarose (VWR International) was dissolved in dH_2O by microwave heating and 6µl ethidium bromide was mixed into the gel liquid, which was set in a mould containing a toothed comb. The 10µl PCR product was pipetted into wells alongside a well containing 5µl 100Kb ladder combined with 1µl 6X loading dye (Promega UK Ltd.). Electrophoresis was performed at 100V for 45 min. Ethidium bromide bound DNA was visualised using an ultra violet (UV) light box and product size determined by reference to the Mw ladder.

2.8.5 DNA sequencing

To ensure genetic specificity of the PCR product, DNA sequencing was utilised to confirm that the primers were annealing to the gene of interest. A 25µl PCR reaction volume was prepared (all the reagents in Table 2.6 multiplied by 2.5) and run on a 2% agarose gel (Section 2.8.4.1). Under UV, the PCR band was cut out using a scalpel blade and placed into an Ultrafree MC-Centrifugal Filter column (Millipore, Durham, UK) which was centrifuged at 7,000 rpm for 5 min. NF H_2O was added to the flow through to bring the volume to 200µl and the DNA product transferred to a clean 1.5ml eppendorf. To each tube, 460µl ethanol, 30µl 3M NaAc (pH 5.2) and 1.5µl glycogen (Roche Diagnostics Gmbh) were added and mixed thoroughly. Samples were placed on dry ice for 30 min, thawed and centrifuged at 13,000 rpm for 15 min. The supernatant was discarded and the pellet washed in 200µl 70% ethanol and spun at 13,000 rpm for a further 15 min. The supernatant was discarded and the

pellet air-dried for 5-10 min before re-suspension in 12µl NF H_2O. The concentration of DNA was measured using a NanoDrop 1000 spectrophotometer (Fisher Scientific Ltd.), where a 260/280nm ratio of ~1.8 was an indication of pure good-quality DNA. DNA was sent to Technical Services at the MRC-Human Genetics Unit, based at the Western General Hospital, Crewe Road, Edinburgh. DNA was sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit and processed on an ABI 3130 Sequence Analyser (Applied Biosystems), as per the manufacturer's instructions.

2.8.6 Quantitative real time - PCR

QRT-PCR was used to determine the absolute amount of a gene present in a sample, relative to an internal control. This method was applied to range of genes in a variety of tissues, all of which had been pre-determined to express the gene by conventional PCR.

2.8.6.1 SYBR Green qRT-PCR

The SYBR Green method for qRT-PCR was implemented in this thesis. SYBR Green is a reporter cyanine dye that intercalates with dsDNA emitting green fluorescence. During the denaturing step of the PCR reaction, dsDNA becomes single stranded DNA and the fluorescence is lost. When the primers anneal to their target and extension occurs, SYBR Green binds the newly synthesised dsDNA strand and the amount of fluorescence emitted is proportional to the quantity of product present in that cycle. The amount of gene is determined by the cycle threshold (Ct) value. The Ct is the cycle at which the fluorescence reaches a certain threshold, above that of background fluorescence, during the exponential phase of the reaction. The PCR cycle is repeated 35 times so that genes with an extremely low abundance can still be detected. To further validate primers for use with SYBR Green chemistries, a standard curve constructed by double dilution (1:2) of an appropriate cDNA template was prepared. A linear curve generating a straight line with an R^2 value near to 1.0 and a slope of -3.33 was desired as this gave an indication of primer efficiency (~100%). An example of one such standard curve is illustrated in Figure 2.1. To ensure primer binding specificity, a dissociation (melting) curve was included, post PCR-cycling, during each run. The plot is the fluorescent signal emitted at the primer melting temperature. A single high peak would indicate binding to one product (gene of interest) therefore discriminating

between non-specific and specific binding. More than one peak would suggest that the primers were binding unintended targets thus invalidating results. An example of a dissociation curve is illustrated in Figure 2.2.

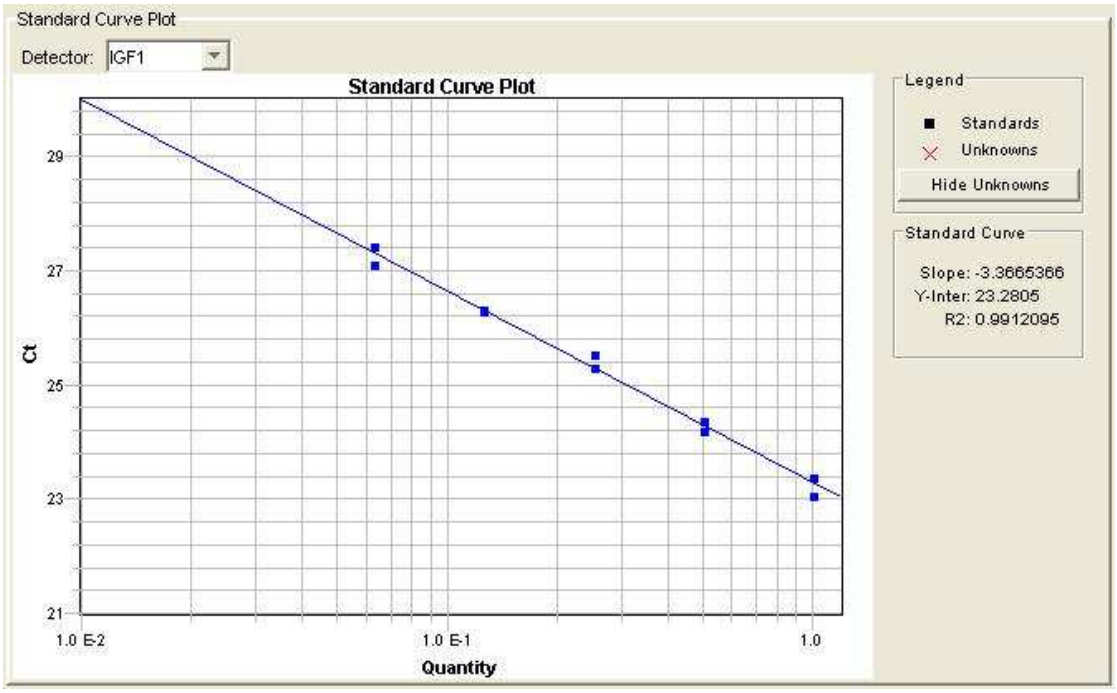

Figure 2.1 A SYBR Green qRT-PCR standard curve. The curve was generated by a series of 1:2 dilutions using adult sheep liver cDNA to test primer efficiency. Primers were designed to bind to the *IGF1* gene.

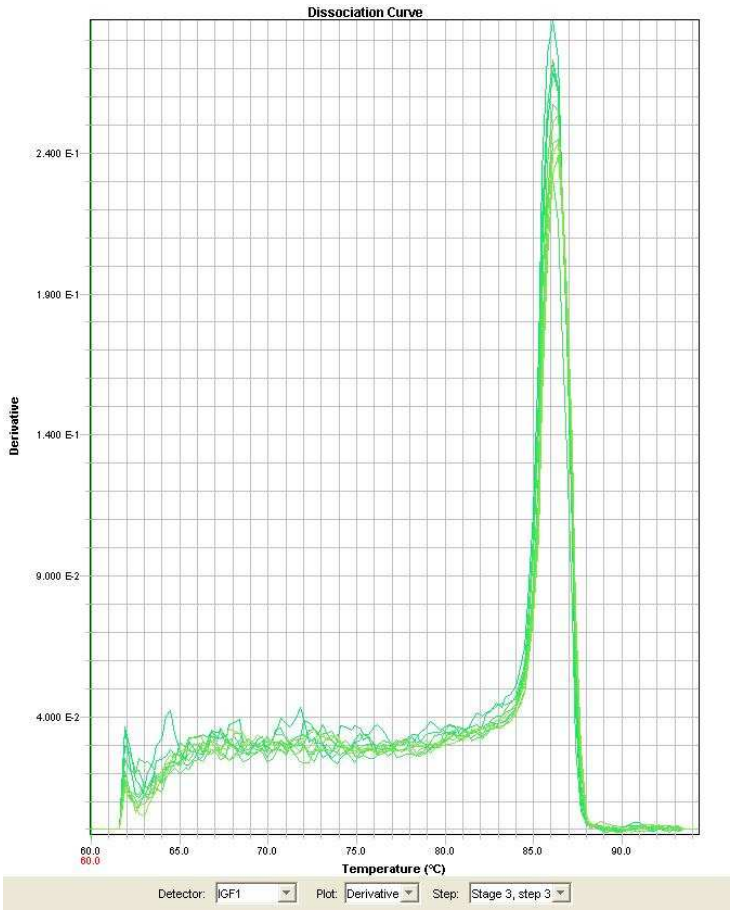

Figure 2.2 A SYBR Green qRT-PCR dissociation (melting) curve. The curve was generated from the same standard curve run for the *IGF1* primers illustrated in Figure 2.1.

2.8.6.2 SYBR Green qRT-PCR protocol

QRT-PCR was performed on either a 96 or 384 well plate format (Applied Biosystems). A 10µl final volume was prepared containing 5µl PowerSYBR Green Master Mix (1X; Applied Biosystems), 0.5µl primer pairs (0.5µM), 3.5µl NF H_2O and 1µl cDNA. The plates were briefly spun to remove liquid from the side of the well, and the plate loaded into an ABI 7900HT Fast Real Time PCR machine (Applied Biosystems). The qRT-PCR cycling programme is detailed in Table 2.8. All samples were run in duplicate and negative controls included a reaction containing cDNA prepared without reverse transcriptase and a reaction replacing cDNA with NF H_20. Each set of samples were run for the gene of interest in parallel with *GAPDH* as an internal control.

Step		Temp. (°C)	Time (min:sec)
Enzyme activation		95	05:00
dsDNA denaturing	X40 cycles	95	00:15
Primer annealing/ strand extension		60	01:00
Dissociation step		95	00:15
		60	00:15
		95	00:15

Table 2.8 The qRT-PCR cycling conditions. PCR was performed on the ABI 7900HT Fast Real Time PCR machine (Applied Biosystems) using the default software settings.

2.8.6.3 SYBR Green qRT-PCR data analysis

Data were compiled using ABI 7900HT v2.3 software and exported to an Excel file. Sample duplicates were checked to ensure precision and the dissociation curve inspected to confirm specificity of PCR product. To analyse raw Ct values the delta (Δ)Ct method was employed to give an absolute expression values relative to *GAPDH* as follows:

ΔCt = Ct of mean *Gene of Interest* – Ct of mean *GAPDH*

Expression relative to *GAPDH* = 2^-(ΔCt)

2.9 Statistical analyses

The majority of data dealt with in this thesis was the comparison of the mean value of raw data between two treatment groups. For example the maternal TP treated animals were compared with the maternal control treated animals. Likewise each fetally treated TP dose was compared back to the fetal control treated cohort. Often there is also reference to the comparison between the maternal and fetal injected cohorts in which the controls groups in each model are compared. Any deviation from this is clearly stated. Statistical analysis was performed using the computational software GraphPad Prism v4.0 (GraphPad Software Inc., San Diego, CA). The data were initially assumed to be normally distributed over the Gaussian bell-shaped curve and an unpaired or paired two-tail Student's t-test was applied as appropriate. In the cases where the data were not normally distributed (calculated by GraphPad Prism) a non-parametric Mann Whitney test was alternatively applied. Intra-group variances were often unequal, which could reflect sample size and also the heterogeneity present in large mammals. Numerical data were presented graphically as the mean ± sem and asterisks used to indicate significant variation between groups. Some analyses in this thesis included the proportional classification of defined categorical groups. These data were subjected to a chi (χ^2) -squared goodness of fit analysis in which the expected distribution of the variable was compared with the observed distribution. P values (*P*) that were <0.05 were considered significant.

Chapter 3

The ovine model of mid-gestation prenatal androgenisation

3.1 Introduction

This thesis describes two models of prenatal androgenisation in the sheep: a novel model of direct fetal androgenisation and a well-established model of indirect maternal androgenisation. These treatment paradigms are based on the method of delivery of androgens to the fetus, one avoiding and one allowing the maternal transfer of exogenous steroids across the placenta. In the direct fetal treated cohorts, fetuses were injected directly with TP on two occasions, either at d62 and d72 or d62 and d82 of a 147-day gestation, and these female offspring were studied as lambs and adults (Fig. 3.1). In a parallel group, pregnant ewes were injected with TP intramuscularly, twice weekly from d62-102 in line with previous studies in the sheep, and are referred to as the maternally treated group. The programming effects in lamb and adult offspring from this cohort were also studied (Fig. 3.1). The effect of prenatal androgenisation was additionally examined in the fetus to investigate the effects of high levels of exogenous androgens during development. Cohorts included female fetuses that were maternally exposed to androgens twice weekly from d60 and assessed at d70 and d90, and fetuses that were directly injected with androgens at d60 and assessed at d70 (Fig. 3.1).

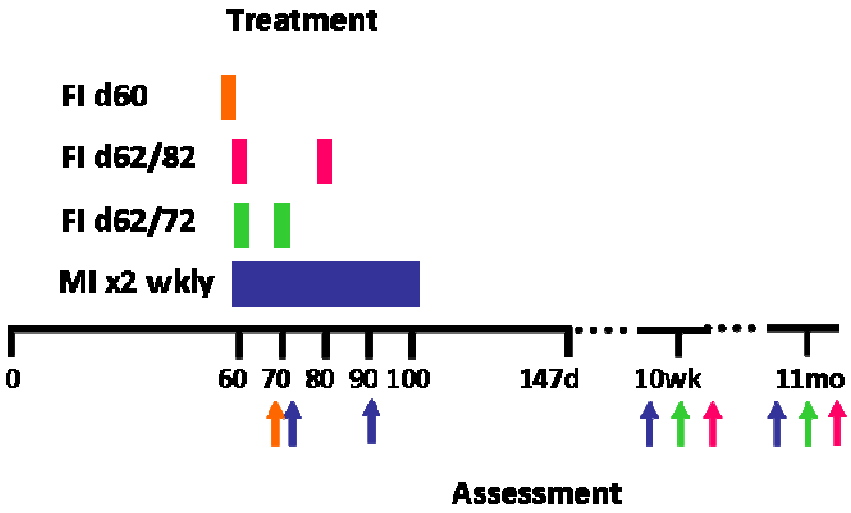

Figure 3.1 The timings of prenatal treatment and prenatal and postnatal assessment of animals. The illustration represents the ovine gestational time-line in days (d) and treatment paradigms during mid-gestation. Offspring were assessed fetally and also at 10 weeks (wks) and 11 months (mo) of age. FI: Fetal injection, MI: Maternal injection.

The rationale for directly injecting fetuses with TP was to avoid the placental aromatisation of this androgen into estrogens, including estrone and estradiol, by the enzyme aromatase. Indeed it has been recently shown that treatment of a pregnant ewe from d30-90 does lead to the elevation of estradiol and estrone levels in the fetus (377), which may have important consequences for the programming of reproductive function in adults (301, 335, 354, 487, 488). Another purpose of developing the direct fetal injection model was to allow manipulation of the dose of androgen to which the fetus is exposed. While many studies have used the non-aromatisable testosterone, DHT, to delineate between the effects of androgens and estrogens in fetal programming (301, 335, 365, 488), the treatment of the mother with either TP or DHT does not allow for dosage control and the amount of steroid reaching the fetus could be variable. A further consideration is the effects that androgens could have on the mother that could indirectly impact on fetal growth and development, such as the induction of insulin resistance in response to elevated androgens (489). The direct fetal injection model therefore avoids any additional maternal influences that are not a direct consequence of elevated androgens.

The fetal injections were timed to coincide with the period of androgen exposure in the maternally treated group. Initial pilot experiments were conducted and found that directly injecting the fetus before d55 of gestation resulted in an unacceptable risk of spontaneous abortion. In addition, injecting directly into the amniotic fluid as a method of delivering the steroid to the fetus was also associated with an unacceptable incidence of abortion. This thesis discusses the effects of direct fetal TP at d62/72 and at d62/82. The d62/72 cohort was developed during preliminary studies that showed that after ten days androgen concentrations were, in fact, still significantly elevated, therefore subsequent studies were conducted with the second TP depot delayed until d82 to prolong exposure. These treatments resulted in an abortion risk of ~1-2% per injection.

In order to measure the degree of mid-gestation testosterone exposure in the developing female fetus, various parameters were examined that are known to be programmed by exposure to the male sex hormone. These included assessment of masculinisation of the genital region and also of the effect of treatment on the placenta and its capacity to metabolise steroids. There is also evidence for the adverse effects of *in utero* steroids on fetal growth, and therefore the effect of androgenisation on birth weight and adult weight was also assessed.

The timing of prenatal androgen exposure is crucial to the development of the urogenital tract (490). The ano-genital distance (AGD) is a sexually dimorphic trait that is a measure of the masculinity of the external genitalia in a newborn, and is about twice as long in males than females (491, 492). The AGD is determined during development by the level of testosterone present in the fetal environment and is therefore a bioassay for this. Manipulation of testosterone concentration during a specific programming window will alter the AGD (490, 493, 494). Accordingly, the AGD was increased in female lambs that were born from mothers that were administered testosterone from d30-90 of pregnancy (377, 495). Androgenisation during this period of sexual differentiation results in the external and internal masculinisation and defeminisation of the female fetus resulting in the development of penis and empty scrotum and the suppression of the lower vagina and opening, although the intra-abdominal development of the ovaries is normal (350, 370, 373, 374, 495). The specific timing of androgen exposure to the masculinisation of the genitals was narrowed down by Clarke *et al*. who showed that treating pregnant ewes with androgens from d30-80 and d50-100 both led to clitoral enlargement in ewes but only the earlier treated group developed a penis and scrotum. Conversely testosterone treatment from d70-120 had no effect on external genital development (370). Aggressive male behaviour was observed in the d30-80 and d50-100 groups but not in groups treated after d70. On the other hand all ewes exposed to increased androgens before d90 exhibited male urinating postures, however, animals treated later from d90-140 did not (370). The concentrations of steroid hormones in the fetal circulation during maternal and fetal treatments were therefore measured to gauge the level of exposure to testosterone, and also to estrogen, and these findings were related to the masculinisation effects of offspring.

The placenta is the site of transfer of steroid hormones from mother to fetus. Androgens can also act directly on the placenta, as previous unpublished work in the lab has demonstrated the expression of AR protein in the placenta. Placental *AR* gene expression was therefore measured to assess whether androgen reception, and therefore action, could be altered by high levels of testosterone in the maternal or fetal environment. Furthermore, the placental expression of genes encoding enzymes that have a role in the metabolism of steroid hormones was studied. Aromatase, which is encoded by *CYP19*, is responsible for the conversion of testosterone into estrogen metabolites (134). The relationship between the expression of placental *CYP19* and fetal estradiol levels and whether this correlated to treatment group, was investigated. In addition, the genes involved in the metabolism of

cortisol were also examined. The type 1 and type 2 11β-HSDs, encoded by *HSD11B1* and *HSD11B2* genes, respectively, are essential for regulating the bioavailability of the glucocorticoid cortisol. Type 1 11β-HSD converts cortisone into cortisol, whereas type 2 11β-HSD acts in the opposite direction, inactivating cortisol (424). Pregnancy, the process of treating the mother or the fetus, and/or the physiologically elevated circulating levels of steroid hormone, could stimulate a stress response. Therefore the placental expression of *HSD11B1* and *HSD11B2* was assessed to determine how the placenta might regulate the fetal exposure to maternal cortisol.

As well as looking directly at the hormonal milieu of the fetus and accompanying placental gene expression, the effect of prenatal androgens on the growth of the fetus was monitored by assessment of birth weight. In girls that are born small for gestational age (SGA) or have intra-uterine growth restriction (IUGR) it has been shown that these individuals have an increased propensity to develop the reproductive anomalies associated with PCOS, including hyperandrogenism, reduced ovulation rate, hyperinsulinaemia, insulin resistance and obesity (496-499). In another study PCOS was correlated with IUGR however while these women were hyperandrogenic they were normoinsulinaemic (500). Conversely other studies have not found any association between SGA and the development of PCOS in adulthood (501, 502). It may be that being SGA or IUGR is associated with these pathophysiological states in the adult rather than being caused by them. SGA or IUGR is more common in babies of women with PCOS (503) and it is possible that the altered maternal steroid environment is responsible for both short-term and long-term effects in offspring. Indeed, it is known that the exposure to glucocorticoids during the perinatal period results in lowered birth weight and metabolic perturbation in adult offspring (277, 278, 286). There may also be species differences related to the effect of elevated *in utero* androgens on fetal growth. In the early prenatally androgenised sheep model in which TP is administered from d30-90 there is increased incidence of IUGR (332, 377, 495). However in the rhesus monkey model, prenatal androgenisation during early or late gestation, did not alter birth weight (504).

The objective of this chapter is to primarily validate the maternal and fetal prenatal androgenisation models. The aims were to study: A) androgen exposure by assessing 1) circulating testosterone and 2) biomarkers of testosterone exposure, B) the role of the placenta including 1) androgen reception, 2) androgen metabolism to estrogen, circulating

estradiol and effect on the fetal pituitary and 3) cortisol metabolism and circulating cortisol, and C) birth weight. By assessing these outcomes in the offspring that were maternally exposed to TP from d62-102, it will confirm that androgenisation has occurred as expected, and that it is in line with other ovine models of prenatal androgenisation. Furthermore, the characterisation of the direct fetal model will allow for comparison between indirect and direct exposure to TP during the same gestational period and may differentiate between predominantly androgenic or estrogenic programming effects.

3.2 Materials and Methods

3.2.1 Experimental animals

The female animals assessed in this chapter are detailed in Table 3.1.

Study	Treatment	Sample number (n)
Fetus		
Fetal Treatment	Injection at d60, assessed at d70	C = 4, TP 20mg = 8
Maternal Treatment	Treatment from d60-70, assessed at d70	C = 3, TP = 6
	Treatment from d60-90. assessed at d90	C = 6, TP = 8
Lamb		
Fetal Treatment	Injection at d62 and 72	C = 6, TP 20mg = 6
Maternal Treatment	Treatment from d62-102	C = 9, TP = 7
Adult		
Fetal Treatment	Injection at d62 and 72	C = 4, TP 20mg = 8
	Injection at d62 and 82	C = 4, TP 1mg = 5, 4mg = 5, 20mg = 7
Maternal Treatment	Treatment from d62-102	C = 5, TP = 9

Table 3.1 The experimental cohorts discussed in this chapter, the treatment regime and corresponding sample numbers.

3.2.2 Hormonal analysis

Fetal blood collected at the time of death was assayed for testosterone, estradiol, cortisol, LH and FSH by RIA. Serum was extracted from the samples as described in Section 2.5.1 and the RIA carried out as detailed in Section 2.5.2 with the specific protocol for each assay documented in Appendix A. The reagent details and CVs are listed in Table 3.2.

Hormone	Primary antibody	Tracer (^{125}I labelled)	Standards	Intra and Inter Assay CVs (%)
Testosterone	Rabbit anti- Testosterone (R3S07-259; AMS Biotechnology, Oxfordshire, UK)	Testosterone (07-189126)	Testosterone (T1268)	4, 8
Estradiol	MAIA Oestradiol Kit (37001; Inverness Medical UK, Cheshire, UK)		Estradiol (kit)	6, 9
Cortisol	Rabbit anti-Cortisol (20-CR5; Fitzgerald, MA, USA)	Cortisol (07-121126)	Cortico-sterone (C2505)	6, 9
LH	In house R29*	Reichart LER-1056-C2-I125 (In-house)	LH NIAMDD-s23	6, 9
FSH	NIDDK-anti-oFSH-I (AFP-C5288113; CA, USA)	NIAMDD oFSH-I (In-house)	NIDDK-RP2 oFSH	7, 11

Table 3.2 The hormones measured in fetal blood by RIA. The primary antibodies, tracers and standards employed for each RIA are listed. Radiolabelled tracer was supplied by MP Biochemicals, Illkirch, France and the standards by Sigma-Aldrich. NIDDK: National Institute of Diabetes and Digestive Diseases, NIAMDD: National institute of Arthritis, Metabolism and Digestive Diseases. *This in-house assay has been described previously by McNeilly *et al.* (505).

3.2.3 Gene expression analysis in the placenta

Genes analysed in the placenta and the forward and reverse primer sequences are listed in Table 3.3. Placental tissue from the fetal side was collected, and RNA extraction, cDNA synthesis and qRT-PCR protocols were followed as detailed in Section 2.8.

Gene (Accession No.)	Forward Sequence	Reverse Sequence	Product Size (bp)
AR (XM_001253942)	GCCCATCTTTCTGAATGTCC	CAAACACCATAAGCCCCATC	233
CYP19 (NM_001123000)	AATCCAGCACTCTGGAAAGC	ACGTCCACATAGCCCAAGTC	152
HSD11B1 (NM_001123032)	ATTCTTGGCCTCATCGACAC	TCCATGATCTTCCTTCCTGG	191
HSD11B2 (NM_174642)	TGTGCCAAGAGCACTACAGG	CTCTACATGTGCCCTGCTCA	120
GAPDH (NM_001034034)	GGCGTGAACCACGAGAAGTATAA	AAGCAGGGATGATGTTCTGG	229

Table 3.3 The primers for genes analysed in the placenta by SYBR Green qRT-PCR. Gene names and accession numbers, forward and reverse primer sequences (5'-3') and PCR product sizes are listed.

3.2.4 Postnatal measurements

The weight (kg) was recorded for each animal at birth, at approximately 10 weeks and 10.5 months of age. The AGD, the distance between the anus and the bottom of the vagina, was measured at 10 weeks and 11 months of age. At 11 months, at the time of sacrifice, a photograph was taken of the genital area and later analysed for the presence of abnormal genital development. The genital area was classed as normal or abnormal with mild, moderate or severe changes of the labial folds and clitoral region. In the most severe cases there was vaginal stenosis and superior fusion of the labial folds. Three investigators independently and blindly classed each photograph into a group based on previously agreed classifications. The vaginal phenotype was also assessed by examining the degree of stenosis in these animals by the vaginal insertion of sponges that are required for cycle synchronisation. Furthermore, masculinisation of the brain was assessed by observation of urinating postures.

3.2.5 Statistical analyses

For the hormonal and gene expression analyses as well as the comparison of body weight and AGD, the difference between two means was assessed, and these data were therefore statistically analysed using a student's unpaired t-test assuming equal variances. In the instances where data were not normally distributed (unequal variances) a non-parametric Mann Whitney test was alternatively used. The values are presented as the mean ± sem. Data collected from the classification of animals by the extent of abnormal genital development were proportions and therefore a chi (χ^2) squared test was performed. These values were presented as a percentage. For statistical tests, P values of $P<0.05$ were considered statistically significant.

3.3 Results

3.3.1 The effect of androgen exposure on circulating testosterone and biomarkers of testosterone exposure

To assess the fetal phenotype in this model, maternally TP-exposed fetuses were collected at gestational d70 and d90, approximately 10 days or 30 days after commencement of twice weekly maternal treatment with 100mg TP. Control animals received an oil (vehicle) injection. At d70 (P<0.05) and d90 (P<0.001) female fetuses had significantly elevated levels of serum testosterone compared to female controls (Fig. 3.2.A). In fact testosterone levels were raised to a comparable level to that of the age-matched male fetus (Fig. 3.2.A). The direct injection of the fetus with 20mg TP resulted in vastly higher circulating testosterone than in the maternal cohort. Serum testosterone levels in females fetally injected with TP were in the order of eighty times greater than female controls (P<0.01) and twenty times greater than age-matched male fetuses (P<0.05; Fig. 3.2.B).

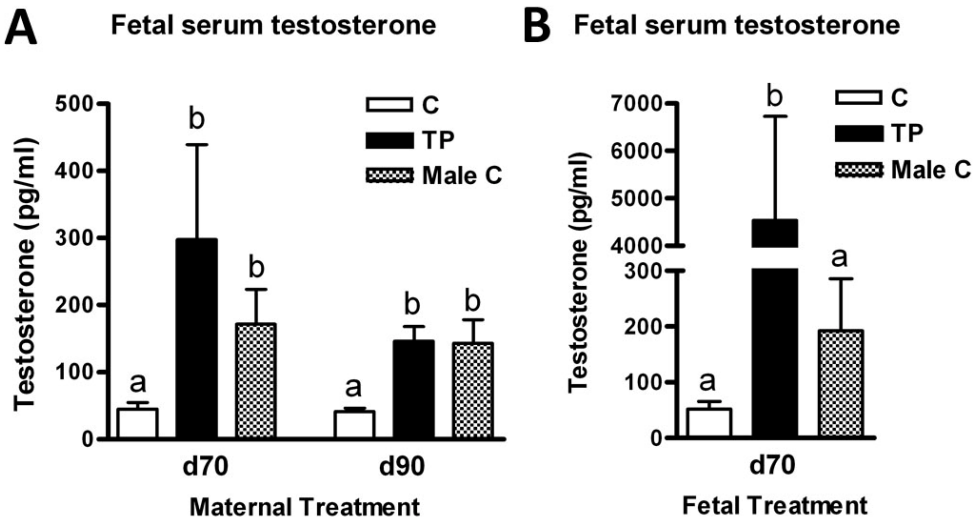

Figure 3.2 The fetal hormonal environment post-TP treatment. Testosterone was measured by RIA in mid-gestation fetuses that were maternally (A) or fetally (B) exposed to TP. Male controls (A; d70 n=8, d90 n=12, B; d70 n=5) are included in A and B as a comparative measure. Different letters represent a statistically significant difference. The data at d70 and d90 in A) were analysed separately.

To confirm that the mid-gestation prenatal androgenisation model did not result in masculinisation of the urogenital tract, the AGD was measured at 10 weeks (Fig. 3.3.A-C) and in the adult ewe at approximately 10.5 months (Fig. 3.3.D-F), and was not found to be altered by mid-gestation exposure of the fetus to androgens in any of the treatment cohorts.

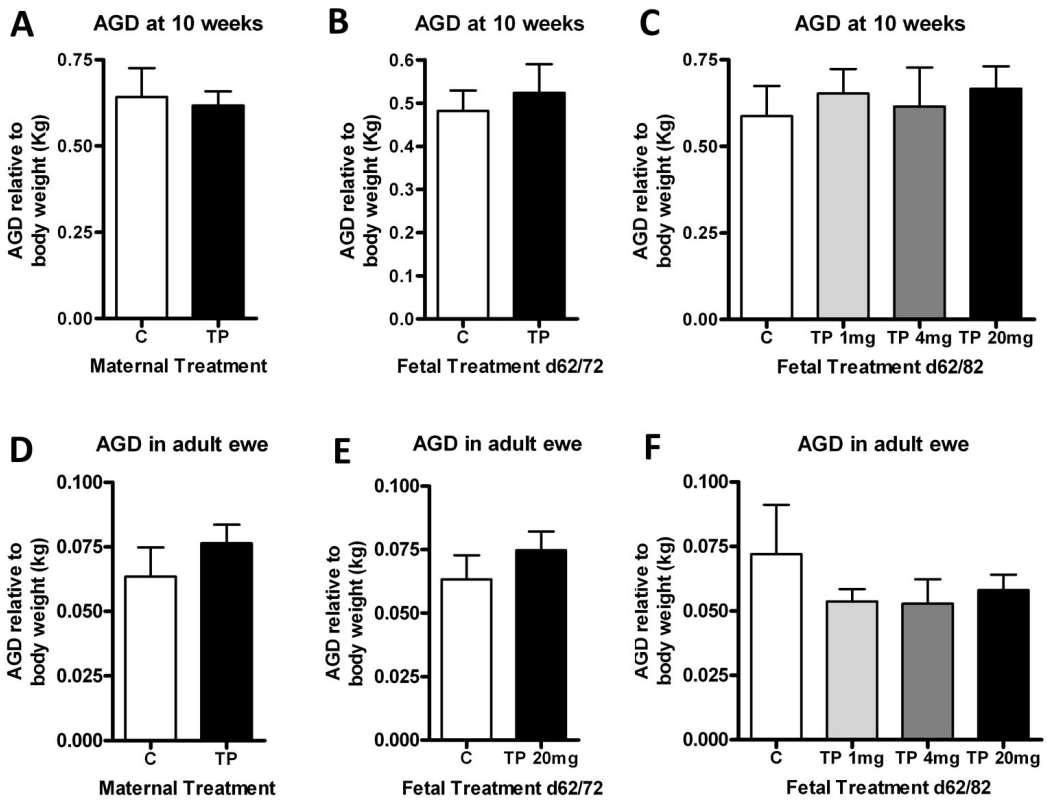

Figure 3.3 The effect of mid-gestation prenatal androgenisation on the AGD of offspring. AGD was recorded at 10 weeks (A-C) and at ~10.5 months (D-E) in maternally and fetally d62/72 and d62/82 treated animals, respectively.

While there were no indications of external masculinisation in the new-born, in terms of AGD, and the external genitalia appeared normal, there was evidence of changes to the labial region of the vagina that were associated with maternal androgenisation in the adult ewe. When TP was administered to the pregnant ewe from d62-102, changes in the development of the genital region were observed in adult offspring including clitoral hypertrophy, some fusion of the labia and stenosis of the lower vagina in the most severe cases. The extent of the abnormality was characterised as mild, moderate or severe in appearance and this is illustrated in the panels in Fig. 3.4.A. At 11 months of age photographs of the genital area were taken and blindly classified as one of the four groups in Figure 3.4.A. Nearly all the

animals (~90%) in the maternally androgenised group were found to have a severe labial and vaginal phenotype using this classification ($P<0.01$; Fig. 3.4.B). In these animals, it was not possible to insert a progesterone sponge for cycle synchronisation, and it would not be possible to mate these ewes naturally.

In the fetally TP-exposed cohorts some animals were also found to possess the severe vaginal phenotype (~25% in the d62/72 TP 20mg group; Fig. 3.4.C and ~20% and ~15% in the d62/82 TP 4mg and 20mg group, respectively; Fig. 3.4.D). The severity of the phenotype was however more variable in these groups with some including animals with mild or moderate abnormalities and some having the appearance of normal genitalia (Fig. 3.4.C and D). It was possible to insert a vaginal sponge in 62.5% of the d62/72 TP 20mg group and 100%, 80% and 71% of the d62/82 TP 1mg, 4mg and 20mg groups respectively. The ewes collectively exhibited some masculinisation of the brain however, as all androgenised animals stood to urinate. The fact that the most severe genital defects occurred in the maternally treated cohort, in which circulating testosterone was significantly lower compared to the fetally injected cohort in which the severe phenotype occurred less frequently, suggests that other metabolites of testosterone may be responsible for the programming of these traits.

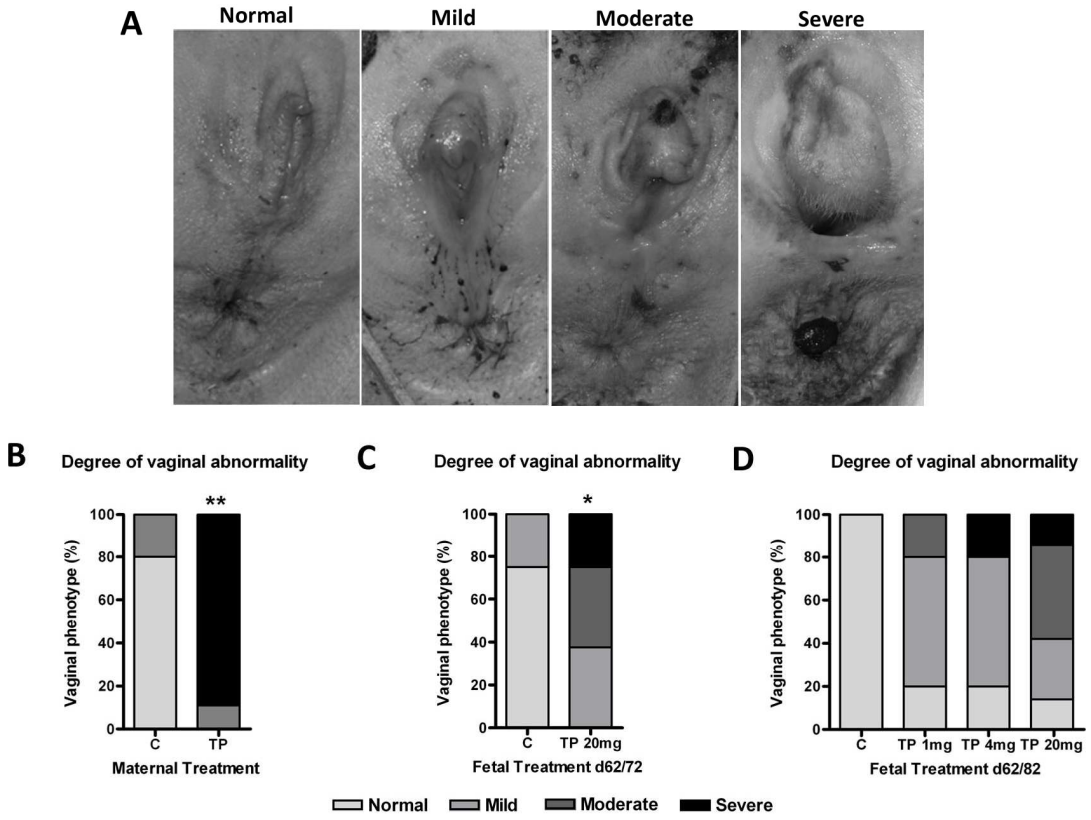

Figure 3.4 The effect of mid-gestation prenatal androgenisation on genital development. In the 11 month ewe anatomical abnormalities in the development of the vagina and external genitalia were characterised as normal, mild, moderate and severe and representative images are shown in A). The degree of vaginal abnormality was semi-quantified by allocation of each animal to a phenotype in maternal (B) and fetal d62/72 (C) and d62/82 (D) cohorts. * $P<0.05$ and ** $P<0.01$.

3.3.2 The role of other steroid metabolites and associated changes in placental function

Fetal estradiol concentrations were measured to assess whether this hormone was altered as a result of increased exogenous TP, arising through the conversion of testosterone into estrogenic metabolites. Estradiol was significantly elevated in the maternally TP-exposed d90 female fetus compared to controls ($P<0.01$; Fig. 3.5.A). This effect was found at d90 only. There was no alteration in the fetal serum estradiol concentration in the fetally TP-exposed d70 female fetuses (Fig. 3.5.B).

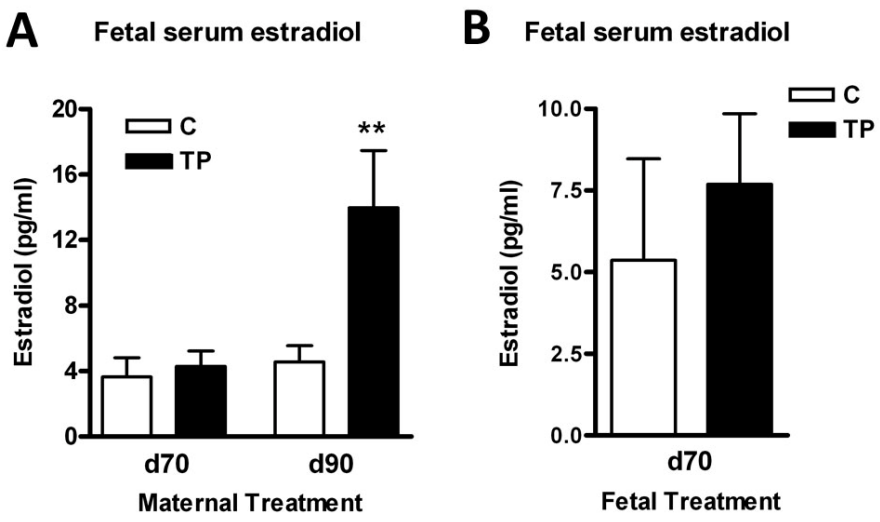

Figure 3.5 The fetal hormonal environment post-TP treatment. Estradiol was measured by RIA in mid-gestation fetuses that were maternally (A) or fetally (B) exposed to TP. ** $P<0.01$.

These findings suggest that the development of the urogenital tract can be affected during mid-gestation through estrogenic programming. This raises the question as to the site of steroid conversion with the most obvious candidate being the placental conversion of testosterone to estradiol, particularly in the maternally treated group in which steroids must cross through this tissue. The receptivity of the placenta to increased testosterone was assessed by measuring *AR* gene expression to identify whether androgen reception, and

hence signalling, in the placenta might be altered as a result of treatment. In addition, the placental expression of *CYP19* was assessed to determine whether the potential for placental testosterone metabolism into estrogens was different in these animals.

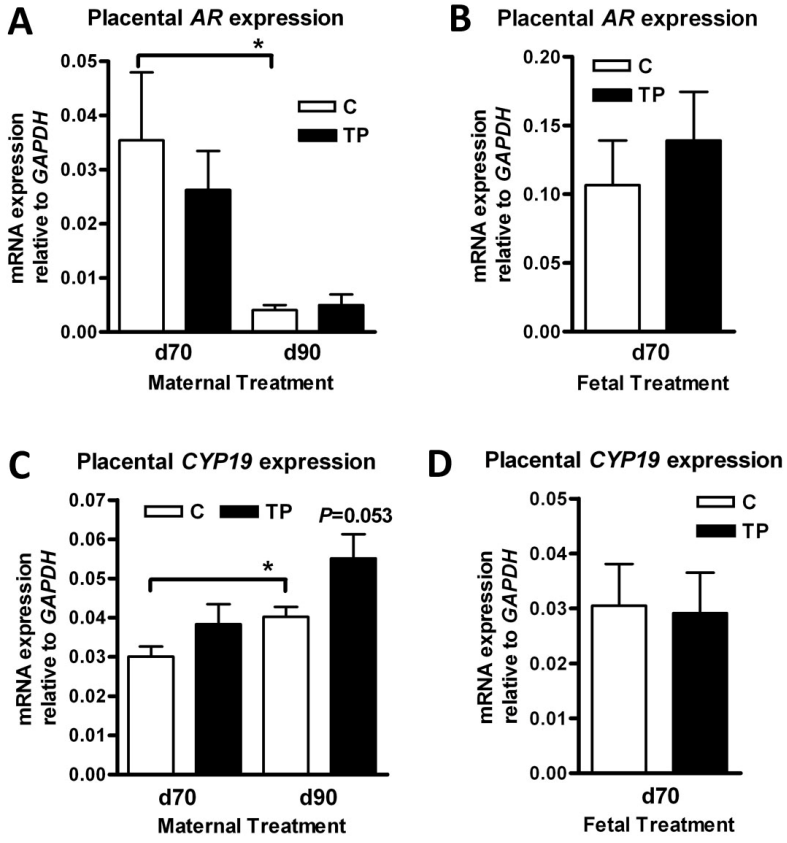

Figure 3.6 Placental *AR* and *CYP19* expression post-TP. *AR* and *CYP19* mRNA expression was measured in d70 and d90 maternally (A and C, respectively) and d70 fetally (B and D, respectively) androgenised fetuses. In A and C the lines represent statistical comparison between control cohorts at two gestational ages. * $P<0.05$.

The placental expression of *AR* was shown to decrease over gestation from d70 to d90 ($P<0.05$; white bars; Fig. 3.6.A). However, neither maternal nor fetal administration of TP altered *AR* mRNA expression in the placenta (Fig. 3.6.A and B), suggesting that androgen reception is not affected by these treatments. In the maternal control cohorts, there was a significant increase in *CYP19* gene expression between d70 and d90 ($P<0.05$), implying that the placenta could have increased aromatase activity at this later gestational age (white bars;

Fig. 3.6.C). In parallel to the pattern of estradiol secretion observed in the d70 and d90 maternally TP treated cohort, *CYP19* expression was not changed at d70 but was increased at d90, however this did not quite reach statistical significance (*P*=0.053; Fig. 3.6.C). Similarly, the expression of *CYP19* remained normal in the placentas of fetally TP-exposed d70 fetuses (Fig. 3.6.D).

The associated increase in fetal estradiol concentrations with urogenital changes in the adult, suggest that estradiol may exert some effect on the developing fetal brain. Therefore an assessment of hypothalamic pituitary function was carried out by measuring gonadotrophin levels. Maternal androgenisation led to a decrease in circulating LH in the d70 and d90 fetus (*P*<0.05; Fig. 3.7.A) however fetal androgenisation did not significantly lower LH levels. Similarly, FSH concentrations were reduced by maternal TP exposure at d70 (*P*<0.05) and d90 (*P*<0.001), but not by fetal TP treatment (Fig. 3.7.B).

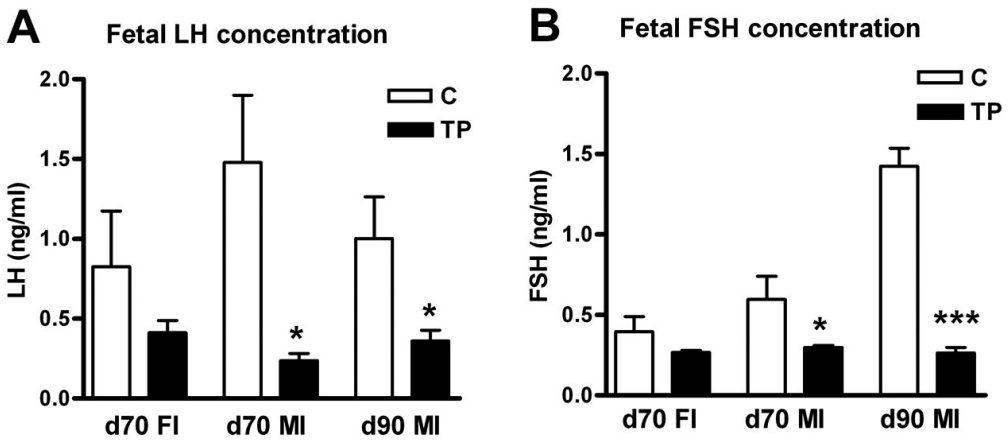

Figure 3.7 Gonadotrophin measurements in the peripheral serum of the prenatally androgenised fetus. LH (A) and FSH (B) were measured by RIA at d70 and d90. **P*<0.05, ****P*<0.001.

In addition to exogenously altering testosterone levels and subsequent effects on endogenous estradiol concentrations in some fetuses, it is also possible that the process of treating the mothers and/or fetuses could inadvertently cause a stress response that would result in changes to fetal cortisol exposure, either directly, or through placental changes. Cortisol was therefore measured directly in the fetus in maternally and fetally treated cohorts, however,

cortisol concentrations were not different as a consequence of androgenisation (Fig. 3.8.A). Interestingly, there was twice as much cortisol hormone present in those fetuses that were fetally injected compared to maternal controls (Fig. 3.8.B) although this was not statistically significant.

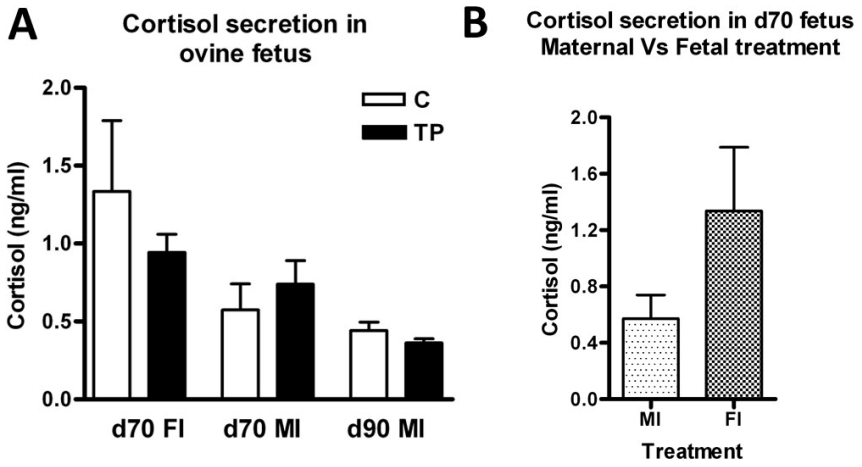

Figure 3.8 Cortisol measurement in the peripheral serum of the prenatally androgenised fetus. Cortisol was measured by RIA at d70 and d90. The effect of maternal and fetal TP exposure was assessed (A) and the amount of cortisol present in the fetal circulation following the fetal control injection was compared with maternal controls (B).

Given that cortisol was measured 10 days after fetal treatment however, this could indicate that cortisol levels were more raised, closer to the time of injection and that injecting the fetus results in elevations of the stress hormone. While this may be due to the responsiveness of the fetal adrenal (Chapter 8) it is also possible that changes in the placental expression of the genes that regulate cortisol bioavailability could be altered. Therefore the expression of *HSD11B1* and *HSD11B2* were assessed. In line with cortisol concentrations, prenatal androgenisation did not alter the placental expression of *HSD11B1* or *HSD11B2* mRNA (Fig. 3.9.A-D) however there was a trend related to the method of TP delivery. The expression of both *HSD11B1* (Fig. 3.9.E) and *HSD11B2* (Fig. 3.9.F) at d70 was higher in fetally injected animals compared to maternal controls respectively. The type 1 isoform was the most abundantly expressed *HSD11B* relative to *GAPDH*, suggesting promotion of cortisol synthesis.

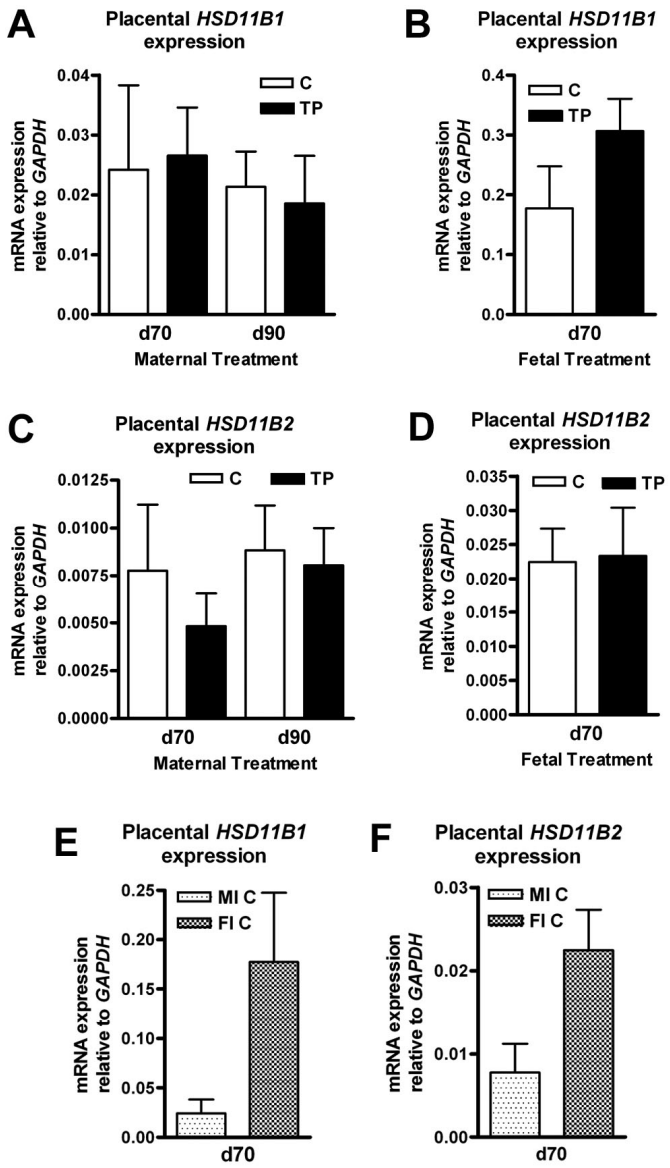

Figure 3.9 Placental *HSD11B* expression post-TP. *HSD11B1* and *HSD11B2* mRNA expression was measured in d70 and d90 maternally (A and C, respectively) and d70 fetally (B and D, respectively) androgenised fetuses. The expression of *HSD11B1* and *HSD11B2* isoforms were compared in control cohorts (E and F, respectively).

3.3.3 The effect of mid-gestation androgenisation on birth weight and post-natal growth

Since increased *in utero* steroids have been associated with IUGR and lowered birth weight, the birth weights of animals were recorded along with body weights in lambs (10 weeks) and in adults (11 months). Exposure of an ovine fetus to TP maternally from d62-102 or fetally at d62 and d72 or d62 and d82 had no effect on birth weight (Fig. 3.10.A-C, respectively), or total body weight at 10 weeks (Fig. 3.10.D-F, respectively) or 11 months (Fig. 3.10.G-I, respectively) of age. There was also no effect of treatment paradigm on the weights at any developmental stage.

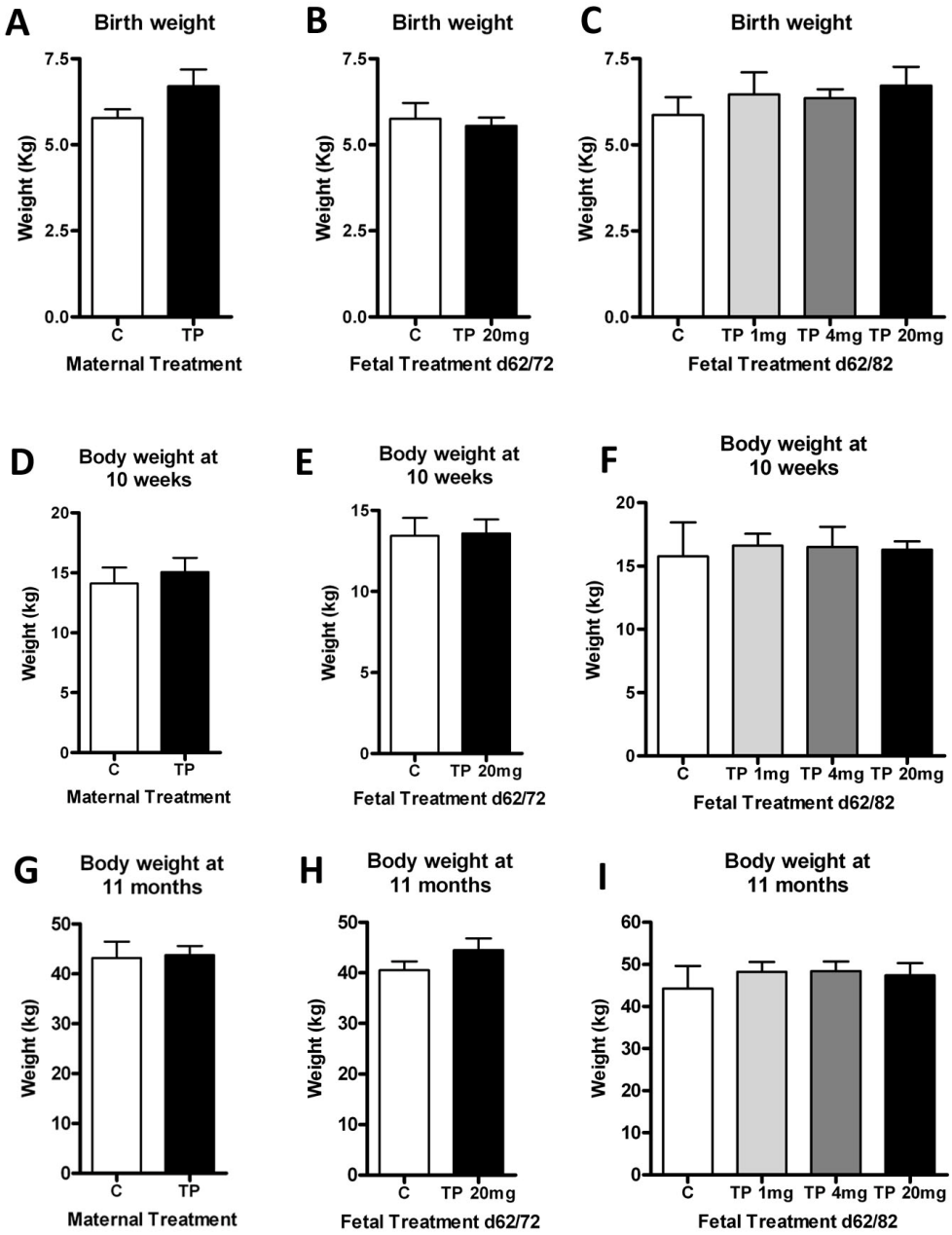

Figure 3.10 The effect of mid-gestation prenatal androgenisation on the weight of offspring. Birth weight (A-C) and body weight at 10 weeks (D-F) and 11 months (G-I) of age were recorded in maternally and fetally d62/72 and d62/82 treated animals, respectively.

3.4 Discussion

Androgenisation of the pregnant ewe with 100mg of TP twice weekly from d62-102 raises the testosterone levels in the female fetus to comparable levels of a control male fetus at the same gestational age (d70 and d90 in this study). This is in line with previously reported fetal circulating testosterone levels in sheep that were exposed for a longer period, from d30-90 of gestation (377). The direct fetal injection model raises female testosterone levels beyond that of a normal male fetus to supraphysiological levels. These animals were assessed at d70, exactly 10 days after the direct injection of 20mg TP. Animals reported elsewhere in this thesis including lambs and adults that received two fetal injections and/or different doses of TP (1mg, 4mg) were not available as fetuses to measure the circulating steroid hormones. We would predict from the considerable levels of TP present in the d70 fetus after ten days that the exogenous exposure is long-lasting and sufficient to span the same period of exposure as in the maternally injected cohort.

The increased conversion of testosterone to estrogen was also confirmed in the maternally TP-exposed cohort (377). This effect was not observed until d90, when the increase in substrate and placental expression of *CYP19*, which encodes the enzyme aromatase, coincided. It is also possible that local conversion of testosterone to estrogen could take place in the fetal tissues including the ovary or liver (506, 507). Nevertheless the huge elevations in testosterone concentrations in the fetally injected cohort did not yield a parallel increase in plasma estradiol concentration implying that the majority of estradiol at this time originates from the transfer of placental metabolites. It was also found that the expression of *CYP19* mRNA was increased over gestational age, which was also associated with a decline in placental *AR* gene expression, suggesting that androgen signalling may have a role in regulating placental aromatase.

A direct bioassay of the extent or timing of fetal androgenisation is the measurement of AGD. Androgenisation from d62-102 occurred after the critical programming window for masculinisation and as expected the AGD in both treatment models were normal. This is in contrast to the increased AGD reported in lambs born to mothers that were androgenised from d30-90 of pregnancy and therefore resulting in a severe masculinised phenotype in their offspring (377, 478, 495). These animals were also born with masculinised genitalia that

included a penis and empty scrotal sac (370, 373, 374, 495). It should be noted however that this severe form of masculinisation is not a feature present in women with PCOS.

A defeminised phenotype was however present in our maternally exposed cohort, in line with previous studies that find clitoral enlargement and abnormal vagina development in sheep exposed during the mid-gestation period (d50-100) without the external masculinisation effect of early androgenisation (370). Very few animals in the fetally exposed cohorts had genitalia that resembled the severe or moderate phenotype pointing towards a mainly estrogenic role of programming. This is supported by the clear evidence of estrogenic programming of reproductive function in women that were prenatally exposed to high levels of the synthetic estrogen diethylstilbestrol (DES). DES was administered to pregnant women to prevent miscarriage, however, in female offspring, it led to reproductive tract abnormalities (508) and a high risk of developing vaginal and cervical cancers (509). In our animals, there may however be some role for testosterone given that the phenotype was not completely absent in the fetally treated cohort. On the contrary, although estradiol was not altered at d70 in the fetally injected cohort, it is possible that variations in estradiol in some animals, due to local conversion of steroids, might programme similar abnormalities as that observed in the maternally treated cohort.

In addition to the physical markers of increased *in utero* androgen exposure, neurological changes were also observed. In sheep, the programming of masculinised behaviour in the brain, also occurs during early to mid-gestation (370). The androgenised ewes in our study adopted male urinating postures, so that instead of squatting, urine was released in a standing position. These animals were easily identified due to their soiled hind region. Although the programming of this masculine behaviour in adult ewes was not quantified they appeared to be common to all prenatally androgenised animals, including the maternally and fetally treated cohorts, implying an androgenic mode of programming.

It is clear that exposing the female ovine fetus to increased androgens mid-way through gestation has subtle effects on urogenital development that does not lead to a fully virilised phenotype, but does result in a partial defeminisation of the genital tract and behavioural traits. These programming effects are likely mediated by the brain and therefore a method to investigate this was to examine the secretion of the pituitary gonadotrophins, LH and FSH. A

defect in this higher centre, that could include the hypothalamus and/or the pituitary, was confirmed by reduced circulating concentrations of LH and FSH following maternal androgenisation. The increased estradiol present in this cohort and not in the fetally androgenised cohort point to estrogenic actions. While estrogen may programme changes in the brain it is also possible that reduced gonadotrophin secretion is a direct effect of negative feedback signalling, on either the hypothalamus and/or the pituitary, by raised levels of estradiol. It has been shown that administration of exogenous estradiol to a sheep fetus at d105 can dampen LH and FSH secretion, however this effect was not found at d90, suggesting that the development of estradiol negative feedback may in fact occur later in development (510). Hypothalamic portal vasculature and gonadotrophin immunopositive gonadotrophs are both present in the ovine fetus by d60-70 of gestation (511, 512) and gonadotrophin secretion is controlled by GnRH signalling (513). The gonadotrophins are known to peak during mid-gestation in the sheep fetus (514) and have been hypothesised to have a direct role in gonadal formation. Inhibition of gonadotrophin secretion by administration of an GnRH agonist does in fact effect testis development but not ovary formation (515). Therefore the effect of lowered gonadotrophins on the mid-gestation female fetus is not known and may not be associated with genital tract formation, but rather highlights the plasticity the developing brain that is a direct target for steroid signalling during this time.

The trend to increased cortisol concentrations ten days after injection in the fetally treated control cohort suggests the induction of a stress response in either the mother or the fetus. Corresponding to this change was the possible dysregulation of the placental *HSD11B* genes which could regulate the transfer of glucocorticoids across the placenta. The type 1 11β-HSD isoform was most abundantly expressed in the ovine placenta at d70 and d90 and although not affected by prenatal androgenisation, expression tended to be up-regulated as a consequence of the fetal injection paradigm when assessed at d70. This implies that a short and uninvasive procedure during pregnancy, that may be associated with maternal anaesthesia or the fetal injection itself, could be accountable for placental changes that alter the mediation of glucocorticoid transport to the fetus. This is an important point that is emphasised throughout this thesis and further illustrates the general plasticity of the fetus to external programming events.

Post-natal parameters were also monitored as an assay of the extent of pre-natal androgenisation of lambs and adult ewes of the two treatment models. Prenatal androgens and cortisol have been linked to reduced birth weight. In humans, there is a higher incidence of SGA or IUGR in babies from women with PCOS (503) and while some reports associate low birth weight with the development of PCOS in daughters (496-500) others do not (501, 516). In non-human primates, prenatal androgenisation during early or mid-gestation does not however lead to reduced birth weight (504). Conversely, in the sheep model, exposure to TP from d30-90 does lead to IUGR and accompanying post-natal catch-up growth (332, 377, 495). In our models, mid-gestation TP treatment from d62-102 did not result in reduced birth weight and growth trajectories into lamb and adulthood were normal. This difference may be a feature of the later exposure of our animals to androgens than in other sheep models.

In the context of previous literature, the maternal androgenisation sheep model is in line with the degree of masculinisation that would be expected to result from the gestational timing of TP exposure, in terms of AGD, external genital development and behavioural traits. It is also found that the treatment of ewes with TP during mid-gestation leads to elevated estradiol levels in the fetus, in addition to testosterone concentration. The fetally exposed cohort, while having pharmacological levels of testosterone in the circulation during mid-gestation, did not possess the same frequency of genital ambiguity as the maternally exposed group. They did however display the same masculinised behavioural traits. All animal birth weights were normal and not affected by prenatal androgenisation and all individuals had healthy growth trajectories. It is therefore concluded that the external programming effects of treatment are minimal and generally reflect that of the subtle masculinised phenotype present in PCOS. The reproductive programming events mediated by increased prenatal exposure to androgens were studied in both fetus and adult offspring. The following chapter assesses the direct and indirect effect of prenatal androgenisation on the fetal ovary.

Chapter 4

The effect of prenatal androgens on the developing ovary

4.1 Introduction

The developing fetus is vulnerable to changes in the hormonal and nutritional environment that can manipulate highly plastic processes and predispose many adult pathophysiologies (517). Dysregulation of fetal programming can lead to an increased stress response as well as a propensity to develop cardiovascular, metabolic and reproductive disorders including PCOS (518-520). While PCOS is present in adult women of reproductive age (162), the early origins of this syndrome may occur in the fetus (350). Evidence from studies in non-human primates, supplemented by experiments in rodents and sheep, reveals that increased *in utero* exposure to androgens leads to perturbed menstrual and estrous cycles, polyfollicular ovaries, hyperandrogenism, increased LH secretion, hyperinsulinaemia and increased adiposity in the adult (297, 365, 477-479). As these traits are reminiscent of PCOS, the androgen-exposed female fetus can inform us about the developmental programming of various features of PCOS.

The effects of maternal androgen administration on the reproductive phenotype of female adult offspring have been well documented; however, these are likely to be secondary to an early direct effect on one or more fetal tissues. The adult consequences of increased prenatal androgens include LH-dependent ovarian hyperandrogenism and hyperinsulinaemia, both of which can cause PCO (521, 522). Therefore it was hypothesised that the adult PCO phenotype is secondary to fetally programmed hormonal and/or metabolic changes, rather than a direct effect on the developing ovary. Thus the aim of this chapter is to assess the effect of *in utero* androgenisation on the fetal ovary at the time of follicle formation.

In fetal life, germ cells migrate into the developing ovary and multiply within oogonial clusters (523). These clusters break down and the germ cells associate with somatic cells to form primordial follicles (524). Concomitantly, there is a marked reduction in germ cell number through apoptosis (525). In the sheep, these key stages of ovarian development peak between days 55-90 of gestation (524). Both fetal and maternal androgenisation investigative approaches, to study the effects of androgens on the developing ovary, were assessed. This study examined the d90 fetal ovary after maternal androgenisation followed by assessment of the d70 ovary. In addition, this was compared to the novel direct fetal androgenisation model at d70, where the fetus was injected with TP at d60 gestation to avoid placental metabolism, as described in Chapter 3.

The effect of androgenisation on cell proliferation and germ cell volume was examined by immunolocalisation of Ki67 and OCT3/4. However, it was also hypothesised that if androgens had a direct effect on the ovary, they would alter the expression of genes involved in pathways associated with ovarian function or development. Key candidate pathways were consequentially assessed: A) the expression of genomic receptors for testosterone and estradiol within the fetal ovary (526), B) the steroidogenic potential of the fetal ovary, by quantifying the expression of key genes involved in ovarian steroid synthesis (159), and C) the ROBO/SLIT pathway that has important roles during mammalian development (527). SLIT1-3 ligands are secreted glycoproteins that bind the ROBO1-4 receptors (527). They can regulate cell migration by acting as a repulsive cue (528) and promote apoptosis and tissue remodelling (529). In the ovine fetal ovary the expression of *SLIT2*, *SLIT3*, *ROBO1*, *ROBO2* and *ROBO4* peak at around d70 coinciding with the onset of follicle formation (530). Importantly, these genes have been shown to be regulated by gonadotrophins and steroids in the female reproductive tissues (527, 531, 532). A final pathway was also examined; D) the expression and localisation of the Ids. The Id1-4 proteins are basic helix loop helix (bHLH) transcription factors that negatively regulate transcription. They are crucial to the timing of tissue-specific gene expression during embryogenesis, by interaction with DNA-binding domains (533) to control growth and differentiation (534). The study of the Ids in this model presented a novel opportunity to investigate their expression for the first time in the mammalian fetal ovary.

Herein the effects of maternal exposure to androgens on the developing ovine fetal ovary at d90 of gestation are described. The effects of maternal androgenisation and direct fetal exposure to increased testosterone on the fetal ovary at d70 of gestation are also quantified. Differential functional alterations that could be early determinants of an adult ovarian phenotype are reported.

4.2 Materials and Methods

4.2.1 Experimental animals

The female animals assessed in this chapter are detailed in Table 4.1.

Study	Treatment	Sample number (n)
Fetus		
Fetal Treatment	Injection at d60, assessed at d70	C = 4, TP 20mg = 8
Maternal Treatment	Treatment from d60-70, assessed at d70	C = 3, TP = 6
	Treatment from d60-90. assessed at d90	C = 6, TP = 8

Table 4.1 The experimental cohorts discussed in this chapter, the treatment regime and corresponding sample numbers.

4.2.2 Tissue collection

Both fetal ovaries (either at d70 or d90) were dissected and one was stored in Bouins fixative for 24 h prior to transfer to 70% ethanol and embedded in paraffin wax while the other was snap frozen and stored at -80°C.

4.2.3 Ovarian histology

The fixed ovaries were processed for histology as described in Section 2.4. Ovary sections (5μm) were stained with H&E to determine their morphology. Immunohistochemistry was performed on fetal ovaries for OCT3/4, Ki67, Id1, Id2, Id3, Id4, AR, ERα and ERβ proteins. Immunohistochemistry was carried out as detailed in Section 2.4.3.1. For negative controls, blocking peptides were available for Id1-4 and AR. For OCT3/4, Ki67 and the ER antibodies, negative controls consisted of omission of the primary antibody and substitution with non-specific IgGs. The product details and dilutions for each of the antibodies used in this chapter are listed in Table 4.2. Immunohistochemistry for the ER antibodies in the fetal ovary required further optimisation due to technical difficulties resulting in poor staining. For these antibodies, a further amplification step was utilised following incubation with the

tertiary compound, using a tyramide signal amplification kit (PerkinElmer, Cambridge, UK). The DNP amplification reagent from the kit was diluted 1:50 with 1X Amplifying Buffer and incubated with the tissue for 10 min at room temperature. The slides were washed in PBS (3 x 5 min). Anti-DNP conjugated to HRP, diluted 1:100 with normal goat serum (NGS), was subsequently added to the tissue for 30 min at room temperature. The tissue was washed in PBS (3 x 5 min) before the routine staining with DAB and the remainder of the protocol performed as previously described. While this antibody amplification method produced immuno-staining, it was very widespread and it was not certain as to the specificity due to the presence of some staining in the controls using this method (Appendix B).

Antigen	Antibody Clone/Source	Dilution	Secondary antibody
AR	Polyclonal Rabbit (N20; sc-816) Santa Cruz Biotechnology Inc., Santa Cruz, USA	1:150	GARB
ERα	Monoclonal Mouse (VP-E614; clone 6F11) Vector Laboratories Ltd., Peterborough, UK	1:30	GAMB
ERβ1	Monoclonal Mouse (MCA1974S; clone PPG5/10) Serotec, Oxford, UK	1:30	GAMB
Id1	Polyclonal Rabbit (C20; sc-488, BP sc-488 P) Santa Cruz Biotechnology Inc.	1:750	GARB
Id2	Polyclonal Rabbit (C20; sc489, BP sc-489 P) Santa Cruz Biotechnology Inc.	1:800	GARB
Id3	Polyclonal Rabbit (C20; sc490, BP sc-490 P) Santa Cruz Biotechnology Inc.	1:200	GARB
Id4	Polyclonal Rabbit (L20; sc491) BP (sc-491 P) Santa Cruz Biotechnology Inc.	1:500	GARB
Ki67	Monoclonal Mouse (NCL-Ki67-MMI), Novocastra Laboratories, Newcastle, UK	1:200	GAMB
OCT3/4	Polyclonal Goat (C20; sc-8629), Santa Cruz Biotechnology Inc.	1:200	RAGB

Table 4.2 The proteins analysed by immunohistochemistry in the fetal ovary. The primary antibodies and respective concentrations used for immunohistochemistry are listed. Secondary antibodies applied were goat-anti rabbit biotinylated (GARB), goat anti-mouse biotinylated (GAMB) or rabbit anti-goat biotinylated (RAGB) at a dilution of 1:500.

4.2.4 Stereological analysis of fetal ovaries

Maternally exposed d90 fetal ovaries were stained for Ki67 and OCT3/4 as described above and subjected to a stereological assessment to quantify proliferation index or germ cell volume, respectively, in control versus TP-treated animals. Stereology was undertaken using Image-Pro Plus 6.2 software combined with a Stereology 5.0 plug-in (MagWorldwide, Wokingham, UK). Each fixed ovary was sectioned through its entirety (5μm sections) and ten evenly spaced tissue sections were studied for each animal. Each tissue section was analysed using the Leitz DMRB microscope (x60 objective) with a Prior Pro-Scan automatic stage attached (Prior Scientific Instruments Ltd., Cambridge, UK) for tiling purposes. Due to relatively few Ki67 positive cells present in the d90 ovary, all positive cells were counted per section. The proliferation index was calculated relative to the average area of each counted section for that individual. Germ cell volume was measured by selecting crosses that overlapped positive OCT3/4 cells in at least five randomly placed grids/fields per ovarian section. Germ cell volume was calculated by dividing the number of crosses selected by the number of crosses per field (432) and multiplied by 100 to give percentage values.

4.2.5 Semi-quantitative analysis of fetal ovaries

Fetal ovaries underwent immunohistochemistry for Id1, Id2, Id3, Id4, and AR as described above and were microscopically assessed to determine changes in protein expression between control and TP-treated animals in both the maternally and fetally treated groups. Staining intensity was classified as weak, moderate or intense to give a measure of expression and fetal ovarian sections were subsequently categorised in a randomised blinded assessment by two investigators with reference to staining exemplars. Variation in staining assessment between observers was <5%.

4.2.6 Quantitative real time -PCR

RNA was extracted from whole fetal ovaries using the Qiagen RNeasy Micro Kit (Qiagen Ltd.) and cDNA synthesised as detailed in Section 2.8.1.1 and 2.8.2, respectively. A number of genes were assessed by qRT-PCR in control and TP-treated cohorts which are listed in

Table 4.3. The protocol for gene expression analysis in the fetal ovary is as described in Section 2.8.6.

Gene (Accession No.)	Forward Sequence	Reverse Sequence	Product Size (bp)
AR (XM_001253942)	GCCCATCTTTCTGAATGTCC	CAAACACCATAAGCCCCATC	233
ERα (NM_001001443)	GAATCTGCCAAGGAGACTCG	CCTGACAGCTCTTCCTCCTG	187
ERβ (NM_001009737.1)	GAGGCCTCCATGATGATGTC	GGTCTGGAGCAAAGATGAGC	208
CYP19 (NM_001123000)	AATCCAGCACTCTGGAAAGC	ACGTCCACATAGCCCAAGTC	152
CYP11A (NM_001093789.1)	CAACGTCCCTCCAGAACTGT	CAGGAGGCAGTAGAGGATGC	172
HSD3B1 (NM_001135932)	GGAGACATTCTGGATGAGCAG	TCTATGGTGCTGGTGTGGA	200
StAR (NM_001009243)	GCATCCTCAAAGACCAGGAG	CTTGACACTGGGGTTCCACT	194
CYP17 (NM_001009483)	AGACATATTCCCTGCGCTGA	GCAGCTTTGAATCCTGCTCT	215
FSHR (NM_001009289)	TAAGCACTTGCCAGCTGTTC	CTCATCGAGTTGGGTTCCAT	196
LHR (NM_214449.1)	TCCGAAAGCTTCCAGATGTT	GAAATCAGCGTTGTCCCATT	199
Id1 (NM_001097568)	TCTGGGATCTGGAGTTGGAG	ATACGATCGTCCGCTGGAA	151
Id2 (NM_001034231)	CATCTTGGACTTGCAGATCG	AGAGAGCTTTGCTGTCATTTG	185
Id3 (NM_001014950)	ACTCAGCTTAGCCAGGTGGA	TTTGGTCGTTGGAGATCACA	160
Id4 (NM_001546)	TCACTGCGCTCAACACCGACC	TTCCCCCTCCCTCTCTAGTGCTCCTG	132
ROBO1 (NM_133631)	TTGAATTCAGGAGCAACTCC	ATTAGCTGCCCTCACAAGGC	161
ROBO2 (NM_001128929)	CTGAGAATCGGGTTGGAAAA	AGGTTCTGGCTGCCTTCTTT	181
ROBO4 (NM_019055)	TGTGAGGCCAGCAACCGGCT	GTGGGCTCTGGGTGGCCCCA	164
SLIT2 (NM_004787)	TGGAGGTGTCCTCTGTGATG	TTATCCTTTCCCCTCGACAA	173
SLIT3 (NM_003062)	GGAGCCTTCACCCAGTACAA	ACACCAGCCCATCAAACAGT	166
GAPDH (NM_001034034)	GGCGTGAACCACGAGAAGTATAA	AAGCAGGGATGATGTTCTGG	229

Table 4.3 Genes analysed by SYBR Green qRT-PCR in the fetal ovary. Gene names and accession numbers, forward and reverse primer sequences (5'-3') and respective product sizes are listed.

4.2.7 Statistical analyses

An unpaired Student's t-test was utilised to compare the means of two groups when analysing the gene expression data. In instances where data were not equally distributed a Mann Whitney test was applied. A chi-squared test was applied to the categorical data gathered from the immunohistochemical analysis of the ovaries. P-values of $P<0.05$ were regarded as significant.

4.3 Results

4.3.1 Maternal prenatal androgenisation does not alter d90 ovine ovary morphology or structure

To investigate the effect of mid-gestation androgen exposure (d60-90) on the ovine fetal ovary a morphological and immunohistochemical assessment was undertaken. The gross morphological structure of the d90 ovary was not altered as a consequence of maternal androgen treatment when assessed microscopically. Cell proliferation was quantified across the ovary by immunohistochemistry for Ki67 (Fig. 4.1.A). Stereological measurements indicated no change in the number of proliferative cells (Fig. 4.1.B). Furthermore, germ cell specific OCT3/4 immunohistochemistry (Fig. 4.1.C) revealed no variation in germ cell volume for control versus maternal TP-exposed ovaries, following quantitative stereological evaluation (Fig. 4.1.D).

4.3.2 Maternal prenatal androgenisation affects d90 ovarian steroidogenic gene expression

The d90 fetal ovary expresses receptors to androgens and estrogens. Maternal androgenisation had no effect on the expression of mRNA for genomic *AR* (Fig. 4.2.A), *ERα* (Fig. 4.2.B) or *ERβ* (Fig. 4.2.C). Functional changes in response to increased *in utero* androgens were however observed in the d90 fetal ovary resulting in gene expression that could significantly impact ovarian steroidogenic potential. QRT-PCR detected reductions in the expression of steroidogenic genes including *StAR* ($P<0.01$; Fig. 4.2.D), *CYP11A* ($P<0.05$; Fig. 4.2.E), and *CYP17* ($P<0.01$; Fig. 4.2.G), but not *HSD3B1* (Fig. 4.2.F) nor *CYP19* (Fig. 4.2.H). Interestingly, *LHR* (Fig. 4.2.I) and *FSHR* (Appendix B), crucial components in stimulating the steroidogenic pathway in ovarian steroidogenic cells in the adult ovary, were also expressed in the d90 ovine fetal ovary, and in the case of *LHR*, was significantly down-regulated following maternal androgen exposure ($P<0.05$).

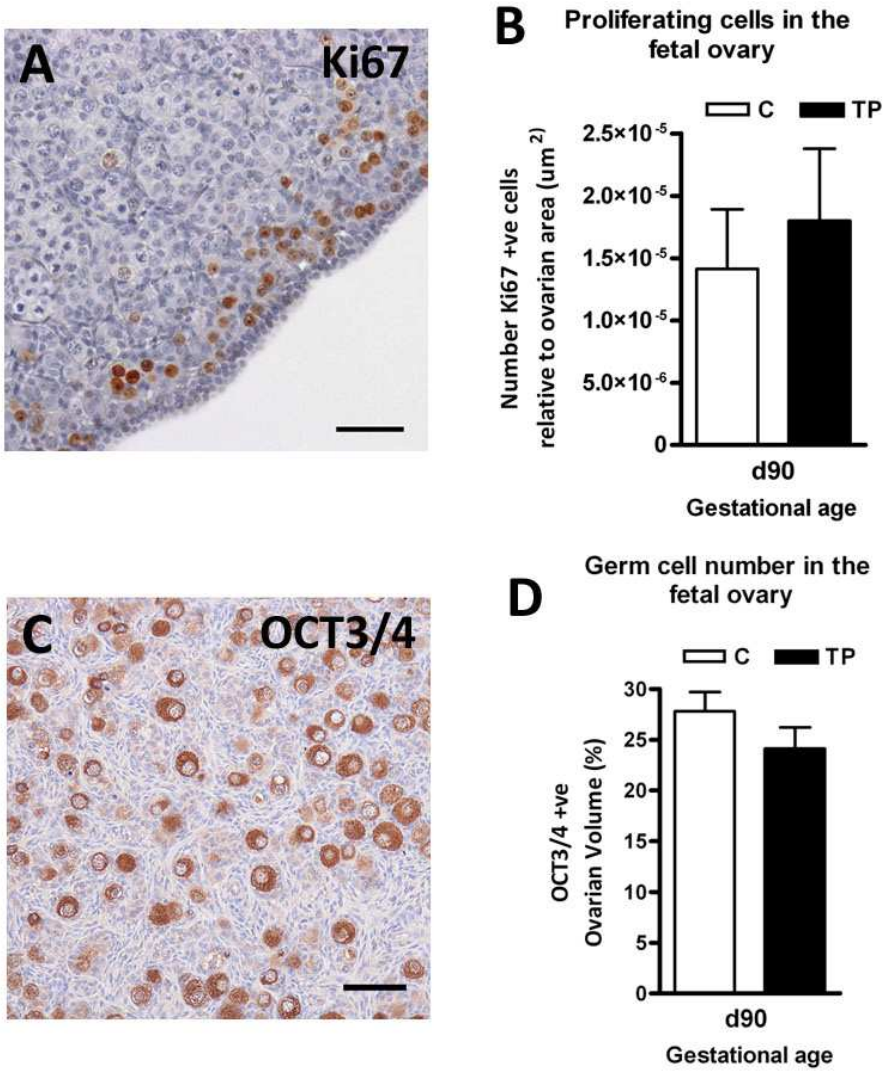

Figure 4.1 The analysis of cell proliferation and germ cell number in the d90 maternally exposed fetal ovary. Immunohistochemistry for Ki67 (A) and OCT3/4 (C) with respective stereological quantification of ovarian cell proliferation (B) and germ cell volume (D) in the maternal TP-exposed d90 fetal ovary. Scale bars = 50µm.

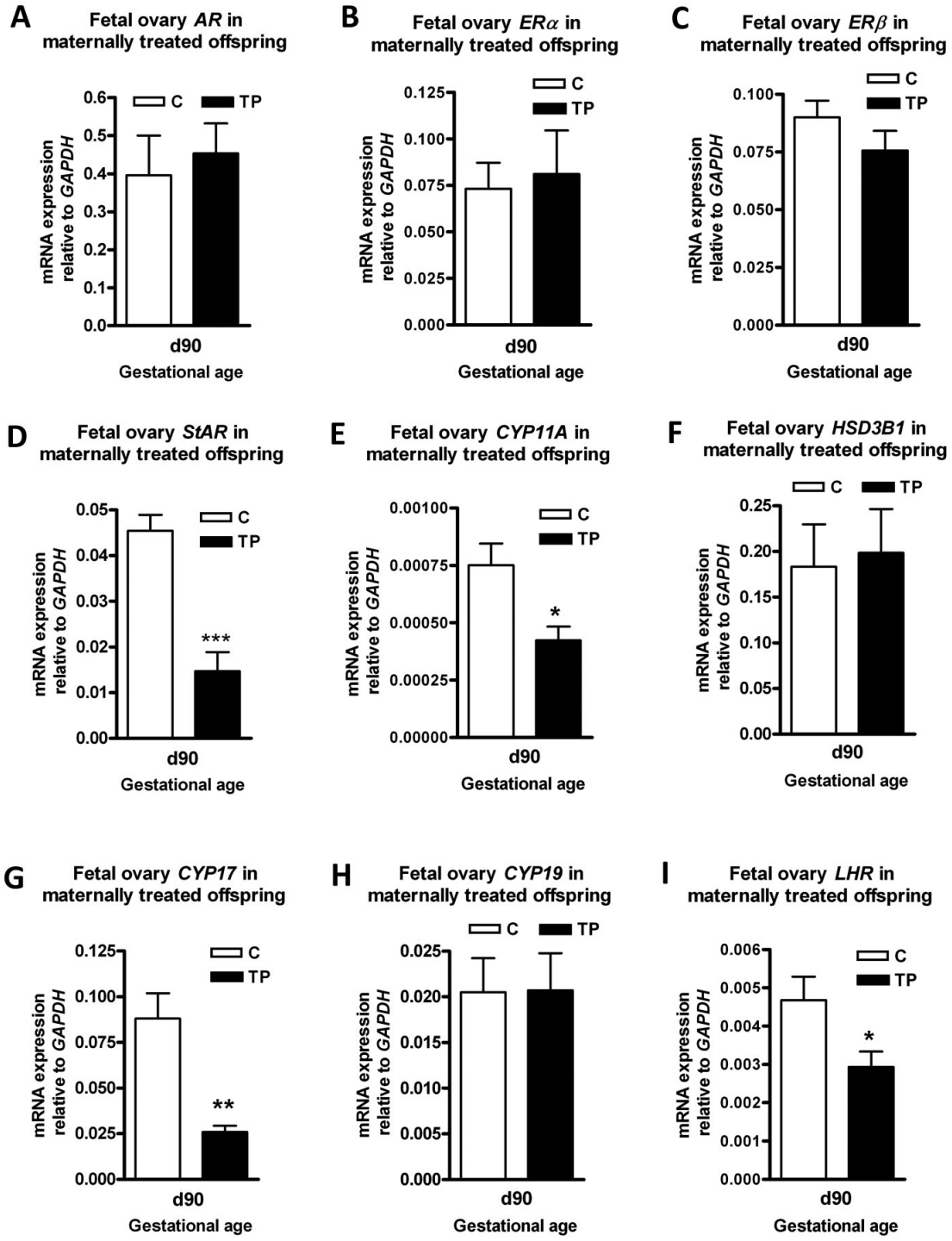

Figure 4.2 Gene expression analysis in the d90 maternally exposed fetal ovary. The effect of maternal TP exposure on *AR*, *ERα*, *ERβ*, *StAR*, *CYP11A*, *HSD3B1*, *CYP17*, *CYP19* and *LHR* (A-I) gene expression in the d90 fetal ovary. * $P<0.05$ and ** $P<0.01$.

4.3.3 Maternal prenatal androgen exposure and other developmental regulatory molecules in the d90 ovary

The effect of maternal androgenisation on the expression of candidate molecules involved in the control of cell proliferation, death and differentiation in the d90 fetal ovary, was assessed. The expression of the SLITs and ROBOs that are expressed during follicle formation in the developing ovine ovary was examined, however; there were no differences in the expression of *SLIT2* or *SLIT3* ligands or *ROBO1*, *ROBO2* or *ROBO4* receptors after maternal androgenisation (Appendix B). All four Id proteins (Id1-4) were expressed in the d90 ovine fetal ovary (Fig. 4.3.A-D). Immunohistochemistry for Id1 revealed protein localisation confined mostly to the germ cells (Fig. 4.3.D). In contrast, Id2 and Id3 protein (Fig. 4.3.A and B, respectively) were strongly expressed in the germ cells and somatic cell streams and Id3 staining was present in pre-granulosa cells adjacent to germ cells (Fig. 4.3.B). Id4 protein expression was present in some but not all somatic cells and pre-granulosa cells and localised to the germ cells (Fig. 4.3.C). While no changes were observed for Id2, Id3 and Id4 mRNA or protein expression following maternal exposure to androgens (Appendix B), staining intensity for Id1 was increased in control versus TP-exposed d90 ovaries in both germ and somatic cell types ($P<0.01$; Fig. 4.3.E), although no variation existed at the transcript level (Fig. 4.3.F). Id1 protein was present in the ovarian stromal cell streams and more strongly expressed in the germ cell cytoplasmic compartment in TP-exposed d90 ovaries compared to controls (Fig. 4.3.D).

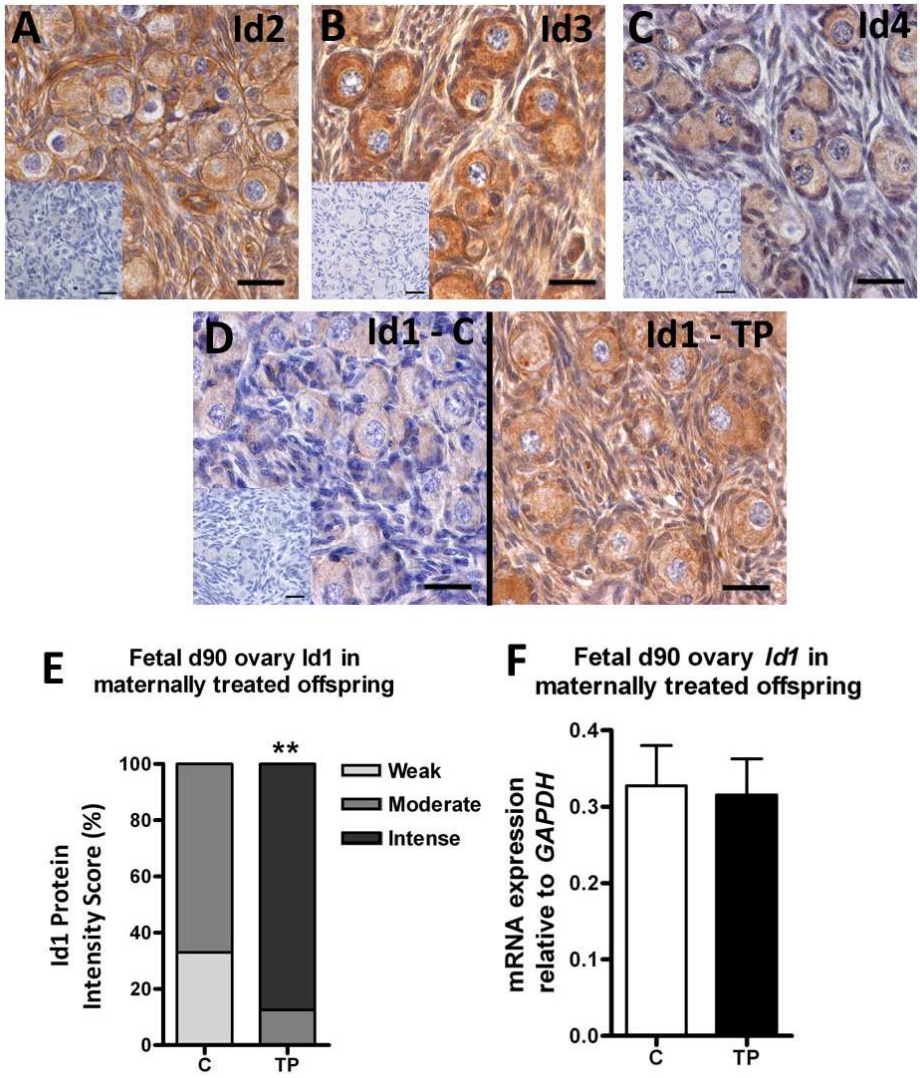

Figure 4.3 Id expression analysis in the d90 maternally exposed fetal ovary. Immunohistochemistry for Id2, Id3, Id4 and Id1 (A-D) in the d90 ovine fetal ovary. Id1 protein expression is altered by maternal TP-exposure (D and E); however, mRNA is not altered (F). Inserts represent negative controls. Scale bars = 50µm. ***P*<0.01.

4.3.4 The effects of maternal and fetal androgenisation on d70 ovarian steroidogenic gene expression

The d70 fetal ovary also expresses receptors for androgen and estrogens. While there were no differences in the expression of *AR* mRNA (Appendix B), immunohistochemistry for the AR protein revealed variations in the level of receptor present (Fig. 4.4.A). Staining intensity for AR was increased in direct fetal TP-exposed d70 ovaries ($P<0.05$; Fig. 4.4.B) but not significantly altered in the maternally exposed d70 ovaries (Fig. 4.4.C). In contrast, direct fetal exposure to TP resulted in a down-regulation of the expression of both *ERα* ($P<0.001$; Fig. 4.4.D) and *ERβ* ($P<0.05$; Fig. 4.4.F) while maternal androgenisation did not alter their expression (Fig. 4.4.E and 4.4.G, respectively).

Maternal androgenisation did not lead to changes in the expression of *CYP11A* (Fig 4.5.A), *CYP17* (Fig. 4.5.B), *CYP19* (Fig. 4.5.C), *StAR*, *HSD3B1*, *LHR* or *FSHR* (Appendix B) in the d70 fetal ovary. Conversely, direct fetal androgenisation did result in alterations to the potential ovarian steroidogenic capacity at d70. The expression of *CYP11A* was decreased ($P<0.05$; Fig. 4.5.D), whereas *CYP17* mRNA was not altered (Fig. 4.5.E) and *CYP19* expression was up-regulated ($P<0.05$; Fig. 4.5.F). There were no changes in *StAR*, *HSD3B1*, *LHR* and *FSHR* (Appendix B) gene expression in this cohort.

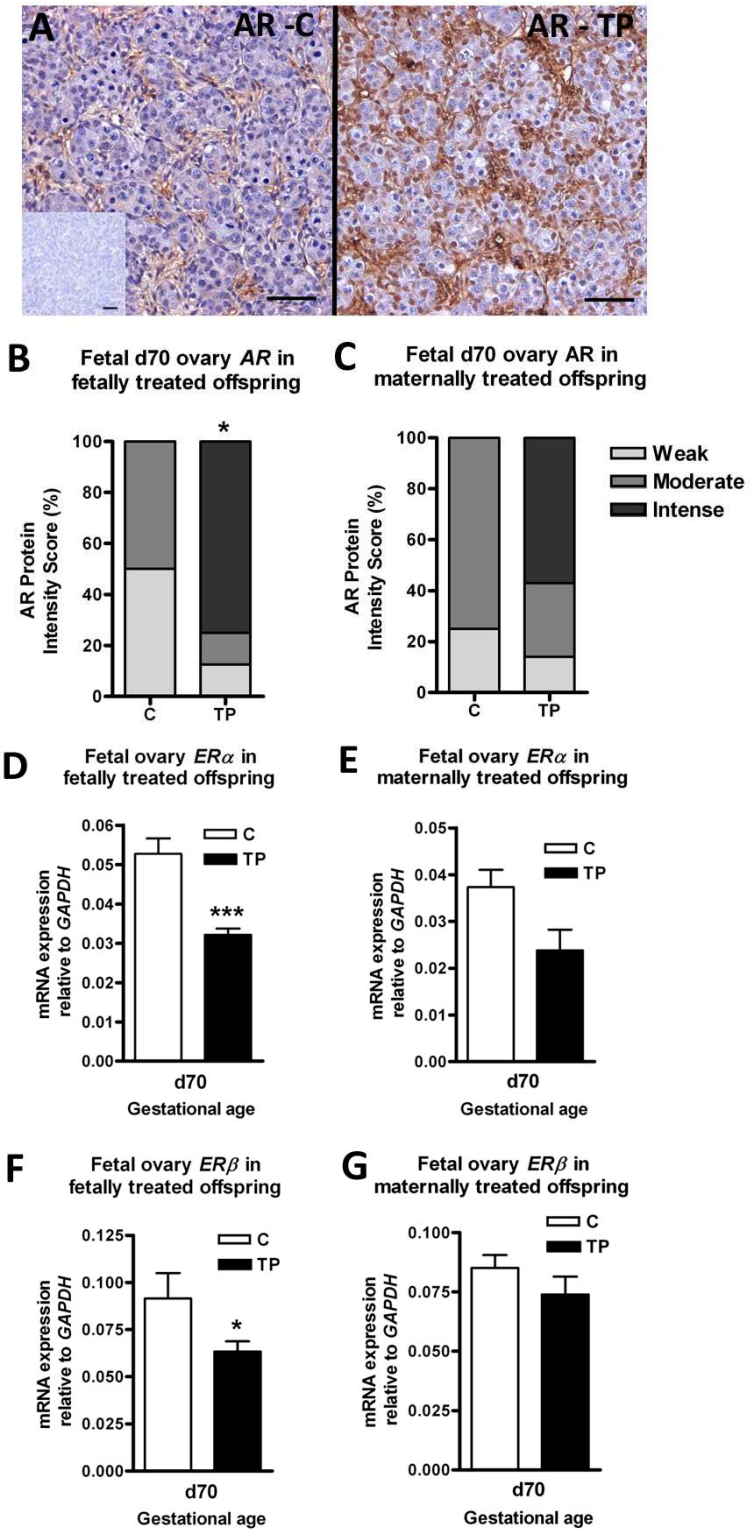

Figure 4.4 Ovarian steroid receptor expression in the prenatally androgenised d70 fetus. Immunohistochemical expression of ovarian AR in a directly treated d70 fetus (A; insert represents negative control), and quantification of the effect of fetal and maternal treatments (B and C). Analysis of fetal and maternal treatment effects on *ERα* (D and E) and *ERβ* (F and G) gene expression. **P*<0.05, ****P*<0.001.

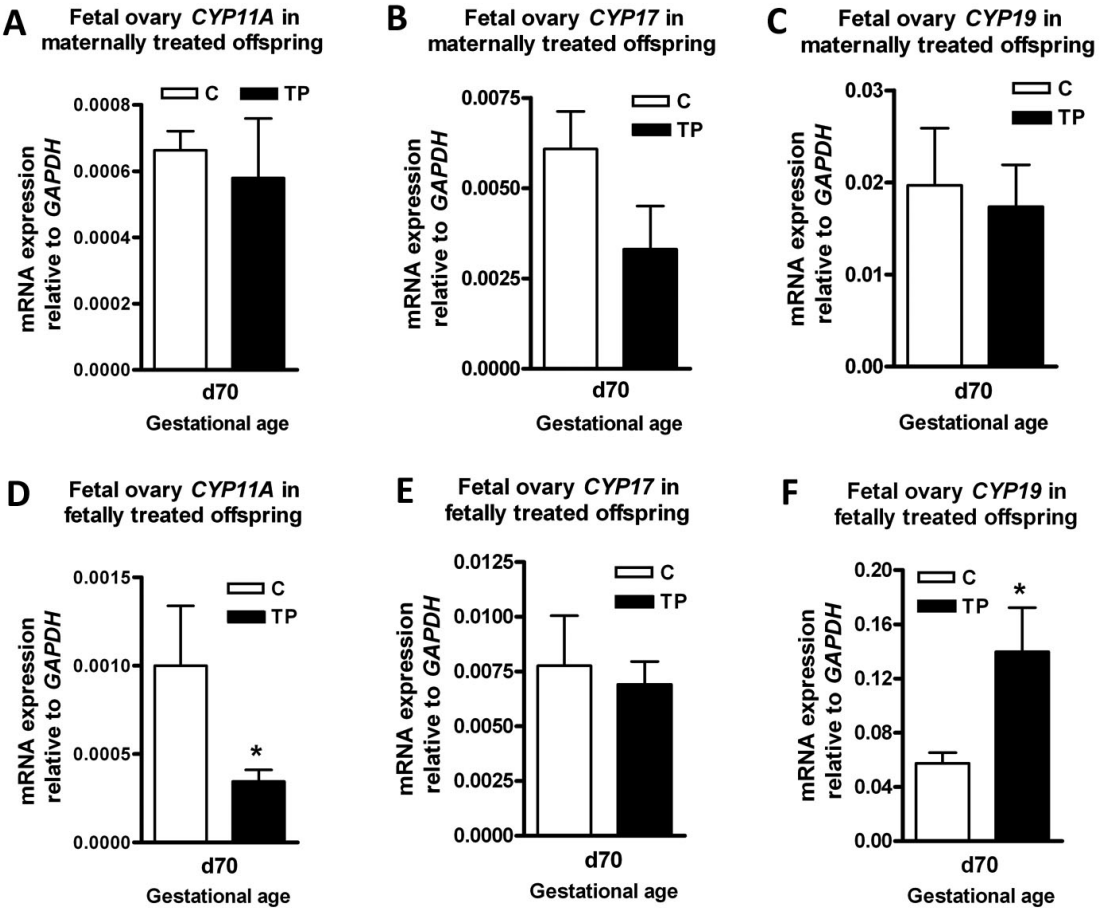

Figure 4.5 Steroidogenic gene expression analysis in the d70 androgen exposed fetal ovary. Effect of TP-exposure on ovarian *CYP11A*, *CYP17*, *CYP19* gene expression in the maternally (A-C) and fetally treated (D-F) d70 fetus. **P*<0.05.

4.3.5 The effect of maternal and fetal androgenisation on other developmental regulatory molecules in the d70 ovary

The *ROBO* and *SLIT* genes are known to peak in the ovine fetal ovary during mid-gestation. No significant changes were identified for *SLIT2* (Fig. 4.6.A) or *SLIT3* (Fig. 4.6.B) mRNA expression due to maternal androgenisation, nor following direct fetal androgenisation (Fig. 4.6.D and E, respectively). Direct fetal androgenisation reduced the expression of *ROBO1* (*P*<0.05; Fig. 4.6.F) while maternal androgenisation had no effect on its expression (Fig. 4.6.C). There were no trends or differences in the expression of *ROBO2* or *ROBO4* after either maternal or fetal androgenisation (Appendix B).

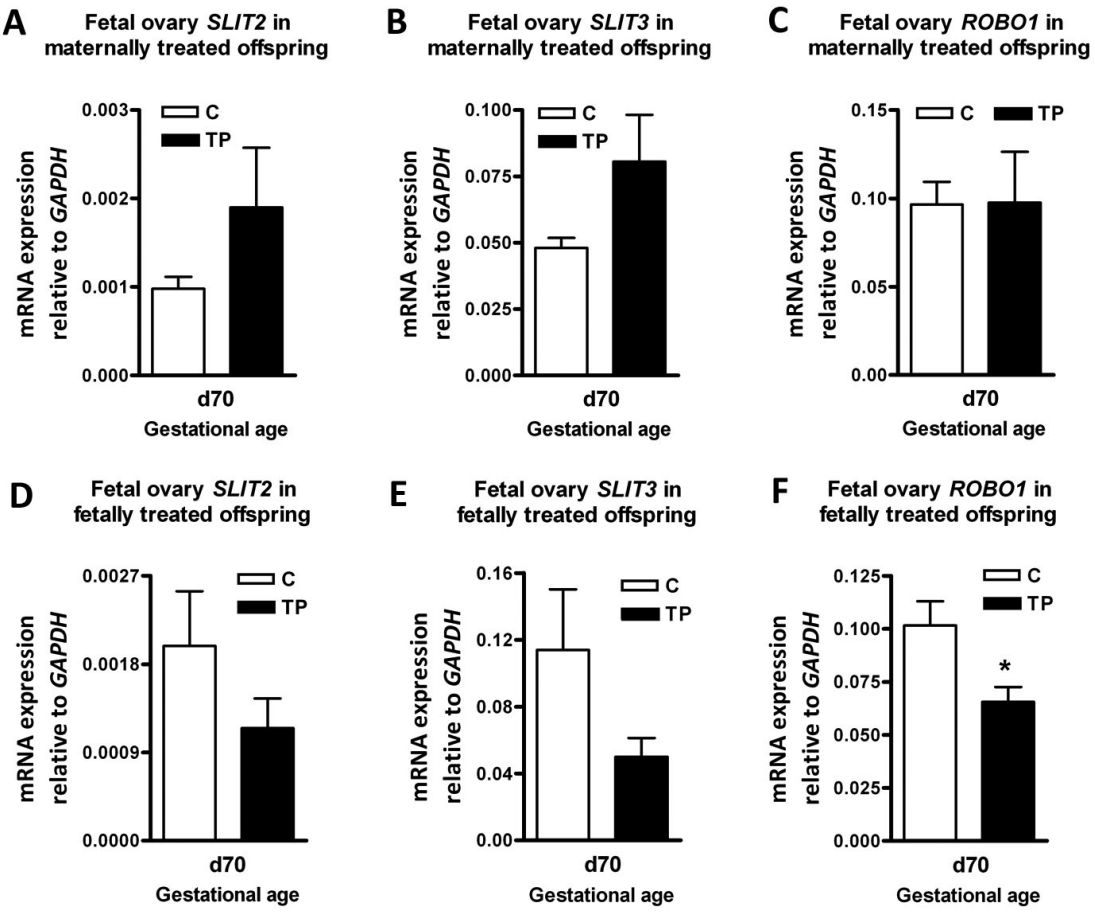

Figure 4.6 ROBO and SLIT expression analysis in the d70 androgen exposed fetal ovary. Effect of TP-exposure on ovarian *SLIT2, SLIT3* and *ROBO1* gene expression in the maternally (A-C) and fetally treated (D-F) d70 fetus. **P*<0.05.

The Id genes were also examined in d70 prenatal androgenisation models. Both mRNA and protein expression for Id3 changed following *in utero* androgen exposure (Fig. 4.7) while no changes were observed for Id1, Id2 or Id4 (Appendix B). *Id3* transcript was increased following direct fetal TP-exposure (*P*<0.05; Fig. 4.7.A), although this trend was not significant following maternal TP-exposure (Fig. 4.7.B). Id3 protein, however, was up-regulated in maternal TP-exposed d70 ovaries, with stronger expression observed particularly in the stromal compartment (Fig. 4.7.C), after both fetal treatments (*P*<0.05; Fig. 4.7.D) and maternal treatments (*P*<0.01; Fig. 4.7.E).

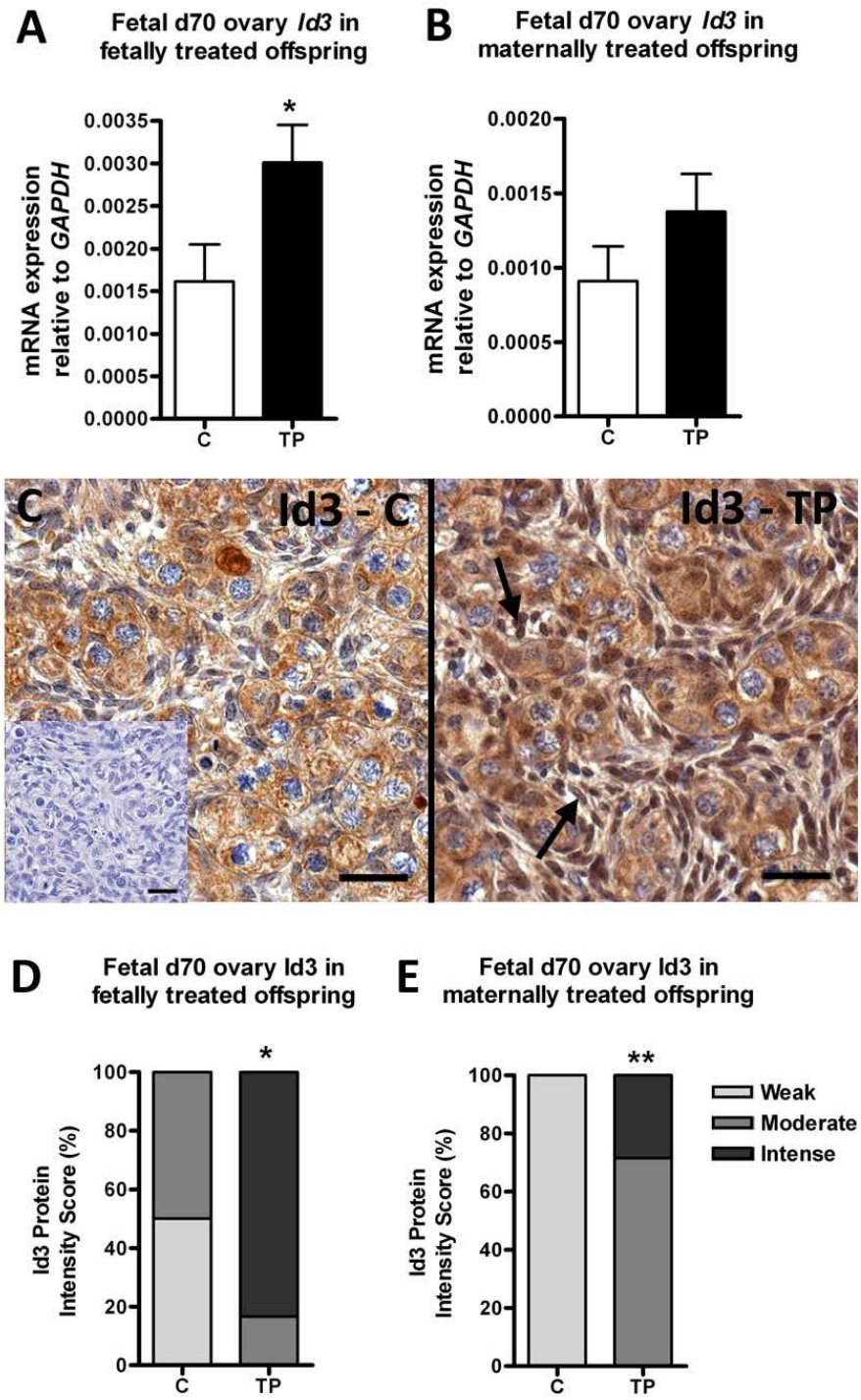

Figure 4.7 Id expression analysis in the d70 androgenised ovary. Expression of *Id3* mRNA in the d70 ovary of fetally (A) and maternally (B) TP-treated animals and a representative image of Id3 protein expression in the d70 maternal control and TP-treated ovary (C; arrows indicate intense somatic cell staining, insert represents negative control). Scale bars = 50µm. Quantification of Id3 protein is illustrated in d70 fetally (D) and maternally (E) treated ovaries. **P*<0.05 and ***P*<0.01.

4.4 Discussion

One key feature of PCOS is the development of PCO characterised by an increased number of small peripheral preantral follicles, an enlarged ovarian volume and thickened stroma (162). It is not clear if the PCO phenotype represents fundamentally different ovaries or if it is an ovarian response to an altered hormonal or metabolic environment. Exogenous or endogenous androgens can make a normal ovary polycystic in appearance (522), as can hyperinsulinaemia (521) and increased LH concentrations (535). These hormonal and metabolic changes are present in women with PCOS (162, 304, 536), and are seen in the ovine model of PCOS (299, 378), so it is possible that PCO occurs in response to the postnatal environment. As prenatal androgenisation in an ovine model leads to both an adult and prepubertal PCO phenotype (301), whether this was reflected in changes at the time of follicle formation in fetal life was investigated.

In women, it has been suggested that since polycystic ovaries contain more growing follicles, without a reduction in primordial follicles, they may contain a larger pool of follicles (316, 330). Given that the follicle pool is set in fetal life, follicle number was investigated in the d90 fetus after maternal androgenisation. At d90 of gestation, around the time of follicle formation, maternal androgenisation had no effects on cell proliferation or germ cell volume. Smith *et al.* utilised a similar ovine model of prenatal androgenisation from gestational d30-90 and also found no alteration in oocyte number at d90, however, by d140 of gestation, there was a significant increase in the number of growing follicles (488). As germ cell proliferation is complete by d90 it is unlikely that there is more germ cell proliferation or follicles formed. It is possible that reduced germ cell loss occurs at later stages of gestation leading to an increased number of follicles at birth. However as the PCO phenotype develops when maternal administration of androgens stops at d90 (299, 355) any effect at later gestational stages may be secondary to effects on other fetal reproductive or metabolic tissues programmed by androgenisation prior to d90.

The mid-gestation sheep fetal ovary has the capacity to be steroidogenically active, expressing a number of enzymatic components of the steroid biosynthesis pathway including *StAR*, *CYP11A*, *HSD3B1*, *CYP17* and *CYP19* (159). Products of these pathways including progesterone, androstenedione, testosterone and estradiol are readily detectable in ovine fetal ovarian tissue homogenates by d75 (159). The role of fetal ovarian steroid production is not

clear but there are ARs and ERs in multiple tissues including the ovary (526). The results showed a significant reduction of *StAR*, *CYP11A* and *CYP17* transcripts in the d90 maternal TP-exposed ovary. As StAR and CYP11A are rate-limiting steps in the steroidogenic pathway (537) the intrinsic alterations following increased exposure to androgens could have a significant impact on the steroidogenic capability of the fetal ovary. In addition, maternal prenatal androgenisation also led to a decrease in d90 ovarian *CYP17* that encodes the enzyme 17α-hydroxylase, responsible for androgen production. Furthermore, the ability of the d90 fetal ovary to respond to circulating endocrine factors was impaired as *LHR* expression was significantly reduced, and this is associated with a reduction in the circulating levels of the gonadotrophins (Chapter 3). Although the consequence of these alterations is not clear, these changes are an indication that the steroidogenic function of the fetal ovary at d90 is altered by maternal androgenisation.

As the mid-gestation fetal ovary matures, cells differentiate, and this is associated with a reduction in germ cell number. The expression of the *Id* genes in the fetal ovary was therefore investigated with the hypothesis that fetal androgenisation could augment their expression to delay some differentiation pathways to potentially alter ovarian cell growth and development. For the first time it is revealed that all four Ids are differentially expressed in germ cells and somatic cells of the fetal ovary at the time of follicle formation. Although maternal androgen treatment had no effect on *Id* expression at the mRNA level there was a striking difference in the immunostaining for Id1 in the d90 fetal ovary. In the fetal ovaries of control animals moderate Id1 staining could be detected in the germ cells, however after maternal androgenisation there was intense staining of the germ and somatic cells. Whether this is a direct or indirect effect of androgen is not known and it is also not clear what functional effect this change would have. Considering its role in other tissues, Id1 may facilitate suppression of subsets of genes required for cell-specific development and apoptosis. The Ids are generally most highly expressed at times when proliferation is high and are decreased in terminally differentiated cells (538, 539). With this in mind it is possible that the up-regulation of Id1 protein reflects further functional changes in the fetal ovary and may delay gene expression necessary for ovarian developmental and maturational processes.

It remains possible that an increase in Id1 at d90 could be a precursor to the increased number of growing follicles present in the d140 ovary, reported previously (488). However

that study, like most previous studies, of the ovine adult phenotype after prenatal androgenisation, commenced maternal androgen treatment at d30 (335, 354, 355, 378, 478). It is clear however that starting treatment on d60 still results in a reproductive phenotype with reduced estrous behaviour (540) as well as hormonal abnormalities, including increased LH pulse frequencies (299). The non-human primate model however is much more characterised and shows a similar effect on menstrual cycles, serum gonadotrophins and androgens, and importantly PCO morphology, when the androgens are started at the early or later stage (298, 358, 477, 541).

The effects of androgenisation on the d70 fetal ovary using both indirect maternal treatment and the novel direct TP model were assessed, avoiding any effects of estrogenic programming that might occur as a result of placental aromatisation. Indeed, although elements of the PCOS phenotype were observed in sheep exposed to the non-aromatisable androgen DHT, there were no clear alterations in ovarian morphology observed in offspring from TP exposed mothers (354), although adult gonadotrophin profiles may still be perturbed (301, 353). The fetuses were injected with TP at d60 and the ovaries were studied at d70 when the circulating testosterone concentrations were still markedly elevated (Chapter 3). The fetal ovary expresses aromatase and it is not known if the intra-ovarian estradiol concentrations are increased after fetal injection of TP. However, there was no difference in the circulating estradiol concentration at this stage in either direct or indirect treatments (Chapter 3). It was therefore possible to compare the fetal ovarian effects of very high concentrations of testosterone (fetal treatment) with a moderate elevation of testosterone (maternal treatment) associated with an adult ovarian phenotype.

The changes in the expression of the steroidogenic enzymes reported at d90 of gestation were not observed at d70 of gestation after maternal androgenisation. While no change in *CYP11A* mRNA expression occurred at d70 with maternal androgenisation, direct fetal exposure to TP did inhibit the expression of *CYP11A*. This may suggest that androgens directly regulate *CYP11A* expression and that it occurs earlier after exposure to higher concentrations of TP. Aromatase was significantly increased in the direct treatment d70 ovaries whereas no change occurred in the maternal exposure cohort. The significance of this in the fetus is not known, nor is it clear if this is a dose effect of the testosterone or whether raised estradiol can inhibit a testosterone-dependent increase in expression.

Direct fetal TP treatment also reduced the mRNA expression of both *ERα* and *ERβ*, a feature not present in the maternally TP-exposed d70 or d90 fetal ovary. This suggests that androgens can affect the ability of the ovary to respond to estradiol. It was not possible to confirm this at a protein level due to non-specific staining in the immunohistochemistry controls (Appendix B) but a previous study has described expression of ERα and ERβ in multiple fetal ovarian cell types including germ cells, surface epithelium, and stromal cells (526). It is therefore possible that TP can directly affect fetal ovary somatic cell function. Although the role of estrogen receptors in the fetal ovary is not clear, while *ER* knockout mice are infertile (542, 543) early reproductive tract development is unaffected (544). However exposure to the synthetic estrogen DES during gestation can result in developmental abnormalities in the gonad and reproductive tract (508, 545, 546).

Unlike *ER* expression, *AR* mRNA was not altered following either maternal or direct fetal prenatal androgen treatment, however, immunolocalisation of the protein revealed an up-regulation of AR in direct TP-exposed d70 ovaries, with a comparable non-significant trend present in maternal TP-exposed d70 ovaries. Administration of DHT to pregnant rats has also been shown to lead to an increase in AR protein in the urogenital tract of female offspring (547). Although the increase in AR protein may be a result of ongoing exposure to high concentrations of testosterone, the fetal ovary appears to be able to adapt to increased androgen concentrations as no changes were found in AR in d90 fetal ovaries. This is in contrast to findings by Ortega *et al*. in a prenatally androgenised ovine model, where treating pregnant ewes with DHT or TP from d30-d90, led to small increases in AR protein in d90 and d140 fetal ovaries (548). It may be that these differences represent a concentration effect or are a result of longer exposure. However, the increase in ovarian AR after direct TP injection in this study, and after maternal DHT (548), suggests an androgenic mode of up-regulation. The lack of agreement between the mRNA and protein expression levels observed in this study may be due to an early mRNA response to increased androgens or changes in mRNA stability or translation or protein turnover. Since the entire frozen ovary was extracted for mRNA additional validation such as Western blotting was not possible.

These observations have revealed that functional changes occur in the fetal ovary as early as d70 and that these are more marked after direct treatment with TP in which there are supra-physiological concentrations of circulating androgen. Similarly, when examining the Ids, Id3 was up-regulated after direct exposure to TP at both the mRNA and protein level. After

maternal androgenisation the trend to increase fetal ovarian *Id3* mRNA was not significant however there was an increase in Id3 immunostaining. Id3 up-regulation occurred in the oocytes and was particularly clear in the somatic cells. This is also consistent with a role for the Ids in fetal ovarian development and indicates that up-regulation of their expression by androgens could have the potential to alter future ovarian development.

In each of the previous examples, with the possible exception of *CYP19* expression, the effects of direct and indirect androgenisation on the d70 fetal ovary have been similar. There was however also a significant change in the expression of *ROBO1* mRNA in the fetal ovary following direct TP-exposure. The SLIT/ROBO pathway can regulate cell migration and promote cell death (527) therefore it was hypothesised that they had an important role in follicle formation in the fetal ovary. Given that there are steroid response elements in the promoter region of these genes (532), and steroids can inhibit SLIT/ROBO expression in the adult female reproductive tract (527, 532), it was suspected that they may be target molecules for androgens in the fetal ovary. The fetal TP-injection did in fact lead to inhibition of the expression of d70 ovarian expression of *ROBO1*. *ROBO1* is the most abundantly expressed ROBO and its localisation to the pre-granulosa cells of forming follicles suggests an involvement in primordial follicle assembly (530). Although the functional effects have not been assessed these findings would be consistent with a direct effect of androgen in inhibiting the ovarian SLIT/ROBO pathway. The nature of the change in *ROBO1* expression in the fetal ovary and the response to different steroid exposure *in vivo* is not clear. However, an alteration in expression of SLIT/ROBO components during the time corresponding to primordial follicle formation, when a finite population of resting oocytes are produced, could have an important impact on adult reproductive success.

While this chapter examines only the fetal ovarian phenotype, prenatal androgen exposure at different time points is associated with changes in adult ovarian function (350, 540). It is also postulated that these are secondary to the hormonal environment, such as increased LH concentrations, and the metabolic environment, with increased insulin concentrations that such fetal androgenisation causes (304, 549). Even in the fetus, in which there are exogenous and subsequent endogenous changes in the steroid environment, other circulating hormones such as the gonadotrophins are affected (Chapter 3) and it is possible that this has indirect roles in reproductive programming. The mid-gestation fetal ovary undergoes marked growth, development and remodelling to form the pool of follicles that will remain throughout the

reproductive lifespan and it expresses both AR and ER (524, 526). Although the morphology of the fetal ovary at d90 gestation is unchanged by exposure to androgens, prenatal androgenisation leads to early subtle, but nevertheless notable functional alterations. The consequences of these changes may alter ovarian development and contribute to an ovarian phenotype in later life. Although estradiol may have a role in manipulating ovarian development it is clear that androgens can have direct effects. However, even in the presence of large concentrations of TP in the pharmacological range, the effects remain subtle.

In conclusion, the PCO phenotype in the adult may not entirely be due to the hormonal and metabolic environment and there may be direct androgen effects on the developing ovary before d90 that programme future ovarian morphology. The following chapters therefore investigate the adult hormonal and ovarian outcomes to determine the primary and possible secondary effects of prenatal androgens. Firstly, a detailed assessment of the Ids in the normal adult ovary and characterisation of the signalling pathways involved is presented, and provides insight into the role of these factors in an adult reproductive tissue.

Chapter 5

The characterisation of Id expression in the adult ovary and their regulation by TGFβ factors

5.1 Introduction

The ovary, unlike other adult mammalian tissues, undergoes persistent cyclical remodelling with regulated proliferation, differentiation and cell death. Introduced in Chapter 4, the Id proteins are a subfamily of regulatory dimeric bHLH transcription factors. These factors regulate many genes, including those required for growth and differentiation, through binding to E-box (CANNTG) sequences on the promoter region of target genes (550, 551). Id proteins lack a basic DNA-binding domain and can heterodimerise with other bHLH proteins to block chromatin binding and thus subsequent transcriptional activity (552). Four known mammalian Id isoforms (Id1-4) have been identified and can regulate growth and differentiation across embryonic tissues (553-555), where both overlapping and non-redundant functions are reported (556, 557). Predictably, these properties have led to Id proteins being implicated in tumourigenesis in various adult tissues (534, 558).

Upstream mediators of Id expression include members of the TGFβ superfamily (559), that include the BMPs and activin (80). Dimeric TGFβ ligands interact with a range of BMP type-1 and type-2 serine/threonine kinase receptor complexes to activate the smad signalling pathway (80). BMPs signal through the smad 1/5/8 pathway whereas activin activates the smad 2/3 cascade which are phosphorylated upon activation by receptor complexes (560), and both are antagonised by smad 6 and smad 7 (561). Signalling by TGFβs has been linked to *Id* gene regulation during development, controlling the timing of differentiation and lineage commitment (557, 562-565). This has particular significance for the adult ovary in which BMPs and activin are involved in many processes governing follicle development and oocyte maturation and competency (255, 566, 567). Furthermore, although not yet characterised in the adult mammalian ovary, *Id* mRNA is reported to be expressed in the hen (*Gallus domesticus*) and the Ids are speculated to be involved in the control and timing of follicle selection and granulosa cell differentiation in this avian species (568, 569).

Although the molecular regulation of proliferation and differentiation in the adult ovary is not fully understood, members of the TGFβ family appear to have key mechanistic roles. The expression of the Id proteins has been studied in the ovine fetal ovary and may be differentially expressed in response to *in utero* programming (Chapter 4). Therefore it was hypothesised that the Ids have roles in the regulation of growth and differentiation of the steroidogenic cells of the adult mammalian ovary. The localisation of Id protein expression

(Id1, Id2, Id3 and Id4) in the pre- and post-pubertal sheep ovary was therefore studied. It was also hypothesised that the Id proteins are differentially regulated by the TGFβ family members through intracellular smad signalling pathways. Accordingly smad proteins were localised in the ovine ovary *in vivo* and the effects of ovarian BMPs (BMP6, BMP15 and/or GDF9) and activin A on the expression of *Id* genes in primary cultures of non-luteinised granulosa cells *in vitro* were investigated. This chapter describes the consistent localisation of the Id proteins in the normal ovine ovary *in vivo* and demonstrates differential gene regulation by BMPs and activin A *in vitro*.

5.2 Materials and Methods

5.2.1 Experimental animals

To characterise the expression of the Ids in the lamb and adult ovary, ovaries from Scottish Greyface lambs (n=12), pregnant ewes (n=15) and non-pregnant ewes (n=5), were collected for immunohistochemical studies. Granulosa cells were collected from a further cohort of Scottish Greyface ewes (n=10) for culture studies. The animals in this study included the offspring of control maternal injections from d62-102 of gestation as well as other animals from additional studies without treatment.

5.2.2 Immunohistochemistry

Tissue was processed for histology as described in Section 2.4.1 and underwent immunohistochemical staining (Section 2.4.3.1) for caspase-3, Id1, Id2, Id3, Id4, phospho-(p) smad 2/3, p-smad 1/5/8, smad 6 and smad7 (Table 5.1). Primary antibodies were incubated with blocking peptide, prior to application, or in the absence of a specific blocking peptide (smad 6 and smad 7) negative controls consisted of incubation with non-specific rabbit IgGs and omission of the primary antibody. To ensure the binding specificity of smad 6 and smad 7, Western blotting was additionally carried out as previously described (Section 2.7), using protein extracted from ovine granulosa cells. Specific bands were visualised for smad 6 and smad 7 at approximately 52 and 42 kDa, respectively, as anticipated (Fig. 5.1).

5.2.3 Immunofluorescence

Id1, p-smad 1/5/8 and smad 6 were visually localised in the ovine ovary by immunofluorescence. Ovary sections were treated in the same way as for immunohistochemistry but for the following alterations to the protocol. Following incubation with the secondary antibody on day 2 and washing, the appropriate tertiary antibody was applied (Table 5.1) for 1 h if using streptavidin or 10 min if using tyramide detection systems (described in Section 2.4.3.2). Sections were subsequently retrieved by microwaving for 2.5 min in 0.01M sodium citrate buffer pH 6.0 (tyramide detection system only) and then

blocked in NGS for 1 h before overnight incubation with the second primary antibody at 4°C. On day 3 the appropriate secondary and tertiary complexes were applied and counterstaining and imaging was carried out as detailed in Section 2.4.3.2.1 and 2.4.3.2.3[1].

Antigen	Antibody Clone/Source	Dilution	Secondary antibody	Tertiary antibody
Cleaved Caspase-3	Polyclonal Rabbit (Asp175, #9661) Cell Signalling Technology Inc., USA	IH 1:100	1	---
Id1	Polyclonal Rabbit (C20; sc-488, BP sc-488 P) Santa Cruz Biotechnology Inc., Santa Cruz, USA	IH 1:1000 IF 1:500	1	A
Id2	Polyclonal Rabbit (C20; sc489, BP sc-489 P) Santa Cruz Biotechnology Inc.	IH 1:500	1	---
Id3	Polyclonal Rabbit (C20; sc490, BP sc-490 P) Santa Cruz Biotechnology Inc.	IH 1:400	1	---
Id4	Polyclonal Rabbit (L20; sc491) BP (sc-491 P) Santa Cruz Biotechnology Inc.	IH 1:200	1	---
p-Smad 2/3	Polyclonal Rabbit (Ser465/46; # 3101) Cell Signalling Technology Inc.,	IH 1:500	1	---
p-Smad 1/5/8	Polyclonal Rabbit (Ser463/465)/(Ser463/465)/(Ser426/428; #9511) Cell Signalling Technology Inc.,	IH 1:50 IF 1:300	2	B
Smad 6	Polyclonal Rabbit (IMG-555), Imgenex Corp, Cambridge BioScience, Cambs, UK	IH 1:100 IF 1:600	2	B
Smad 7	Polyclonal Rabbit (IMG-531), Imgenex Corp.	IH 1:100	1	---

Table 5.1 List of primary, secondary and tertiary antibodies used immunohistochemistry (IH) and immunofluorescence (IF) experiments. Secondary antibody; 1) GARB (Dako, Glostrup, Denmark) 1:500, 2) Goat anti-Rabbit Peroxidase (Dako) 1:200. Tertiary antibody; A) Streptavidin Alexafluor 546 (s-11225 Invitrogen, Paisley, UK) 1:200, B) Tyramide Fluorescein (Perkin Elmer LAS, Inc. MA, USA) 1:50.

[1] Immunohistochemistry and immunofluorescence undertaken for this chapter was carried out, in part, by Sophie Etherington under supervision by KH.

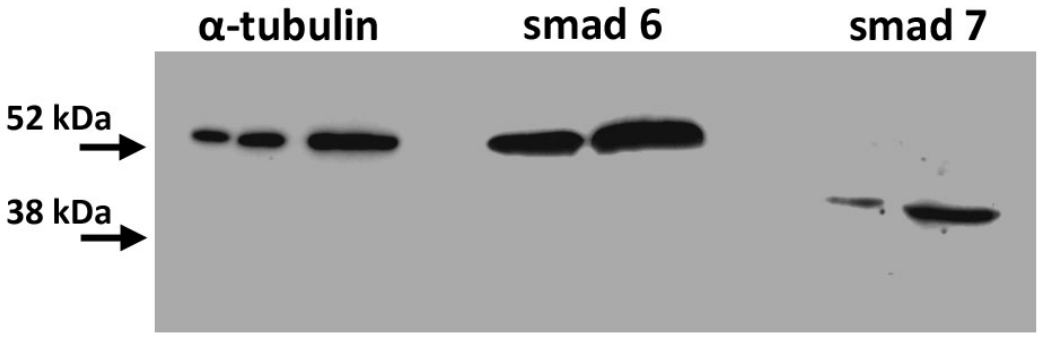

Figure 5.1 Validation of smad 6 and smad 7 immunohistochemical staining specificity by Western blot. In each lane 20µg of protein was loaded. Primary antibodies applied were α-tubulin as an internal control (1:4000; Monoclonal mouse anti- α-tubulin, T9026; Sigma), smad 6 (1:300, Polyclonal rabbit anti- smad 6, IMG-555; Imgenex Corp.) and smad 7 (1:800; Polyclonal rabbit anti smad 7, IMG-531; Imgenex Corp.).

5.2.4 Quantification of thecal Id expression in healthy and atretic follicles

To obtain a quantitative measurement of thecal Id expression in follicles, staining intensity for Id1, Id2, Id3 and Id4 proteins were classified in three groups: absent, partial or intense stain. Follicles were classified as atretic or non-atretic based on immunostaining for activated caspase-3 in a serial section. Forty non-atretic and forty atretic follicles (50:50 pre-antral and antral) were examined across 6-8 different ovaries and a count performed categorising follicles into the appropriate staining class based on clear visual reference pictures agreed by three observers. Proportional data were presented as a percentage of the total number of follicles examined in each group.

5.2.5 Cell culture and quantitative real time PCR

Granulosa cells were collected and cultured as previously described (570). Follicles (<3.5mm) were collected from ovaries of ten Scottish Greyface ewes during the estrous cycle. Follicles were hemisected and the granulosa cells collected by flushing thecal shells using a 1ml syringe. The supernatant containing granulosa cells was removed and cells collected by centrifugation, washed, and re-suspended in culture medium (McCoys 5a medium with sodium bicarbonate, supplemented with 0.1% BSA, 0.5x PenStrep, 3mM L-

glutamine, 5µM transferrin, 0.3mM testosterone, 4nM selenium, 0.01µM insulin, 0.01µM ovine IGF-1 LR3 (Novozymes Biopharna AU Ltd, Australia), and 1nM FSH (NIDDK). Three separate experiments consisted of pooling granulosa cells from 3-4 animals to obtain sufficient numbers. Approximately 75,000 granulosa cells per well were cultured in 200µl medium with the addition of 100ng/ml activin A and/or BMP6, BMP15 and/or GDF9 (R&D Systems, Abingdon, UK) in treatment wells, or carrier-only in control wells, at 37°C/5% CO_2 for 24 h[2]. These concentrations were chosen based on the existing literature regarding BMP and activin effects on ovine steroidogenic cells (571, 572).

Cells were lysed in 50µl RLT Buffer containing 1% β-mercaptoethanol and one volume of 70% ethanol was added and the contents mixed and transferred to an RNeasy MiniElute spin column (Qiagen Ltd.). RNA was extracted using the Qiagen RNeasy Micro Kit as per the manufacturer's guidelines (Qiagen Ltd.). Lysed cells from 2-3 wells were pooled in order to obtain adequate RNA concentration and purity (A_{260}/A_{280} ratio) which were measured using a NanoDrop 1000 spectrophotometer (Fisher Scientific Ltd.) as described in section 2.8.1.3. RNA was stored at -80°C until cDNA was synthesised from 200ng total RNA per reaction as described in Section 2.8.2 using the High Capacity cDNA Reverse Transcription Kit (Applied BioSystems). Primer sets were designed for amplification of partial regions of target genes by qRT-PCR (Table 5.2) and qRT-PCR was performed. The full protocol was followed as described in Sections 2.8.3 and 2.8.6.

[2] Granulosa cell collection and culture was performed by Dr Julia Young

Gene (Accession No.)	Forward Sequence	Reverse Sequence	Product Size (bp)
Id1 (NM_001097568)	TCTGGGATCTGGAGTTGGAG	ATACGATCGTCCGCTGGAA	151
Id2 (NM_001034231)	CATCTTGGACTTGCAGATCG	AGAGAGCTTTGCTGTCATTTG	185
Id3 (NM_001014950)	ACTCAGCTTAGCCAGGTGGA	TTTGGTCGTTGGAGATCACA	160
Id4 (NM_001546)	TCACTGCGCTCAACACCGACC	TTCCCCCTCCCTCTCTAGTGCTCCTG	132
GAPDH (NM_001034034)	GGCGTGAACCACGAGAAGTATAA	AAGCAGGGATGATGTTCTGG	229

Table 5.2 Cultured granulosa cell gene analysis using SYBR Green qRT-PCR. Gene names and accession numbers, forward and reverse primer sequences (5'-3') and PCR product sizes.

5.2.6 Statistical analyses

For the quantification of Id protein expression in the ovary, a chi squared test was applied to analyse whether Id expression was associated with healthy follicles. For the gene expression analysis data, the relative expression level of each target gene to *GAPDH* was quantified using the ΔΔCt method (fold difference) so that control data were standardised between qRT-PCR runs to 1.0. Data are presented as the mean ± sem and statistical analysis was performed using a one way analysis of variance (ANOVA) with Bonferroni pair-wise comparison. P values of $P<0.05$ were regarded as significant.

5.3 Results

5.3.1 Id proteins are expressed in ovine follicles

Id proteins were localised in sheep ovaries from different functional stages including pre-pubertal, pregnant and non-pregnant states. Specific immunostaining was observed for Id1, Id2, Id3 and Id4 in the follicles of all ovaries examined (Fig. 5.2). These were consistently localised to granulosa cells in follicles throughout development from primary to pre-ovulatory stages. In addition, consistent weaker staining was observed in the theca cells of these follicles (Fig. 5.2). Id2 immunostaining was also apparent in the oocytes at all follicular stages and there was strong antibody binding observed across the ovarian stroma (Fig. 5.2.D-F). Weak Id3 expression was visible in the oocytes of primordial follicles (Fig. 5.2.G; black arrows) whereas the zona pellucida exhibited a high level of Id3 expression across follicle development (Fig 5.2.H and I). Specific immunostaining was also observed in endothelial cells for each Id protein examined (Fig. 5.2), particularly Id3 where there was strong blood vessel localisation of this protein (Fig. 5.2.G; grey arrows).

5.3.2 There is differential localisation of Ids in granulosa cells

The Id1 antibody revealed intense staining of peri-oocytic cumulus cells (Fig. 5.2.A-C), a striking observation that was reproduced across follicular development and in all specimens, including pre-pubertal lamb and pregnant sheep ovaries as well as cycling ovaries. Although Id4 was more intensely immunostained than Id3 their localisation was similar across the granulosa cells and was maintained throughout follicle development (Fig. 5.2.G-L). However, unlike Id1, Id3 and Id4, immunoreactivity for Id2 was consistently most intense in those mural granulosa immediately adjacent the antral cavity of tertiary follicles (Fig. 5.2.F).

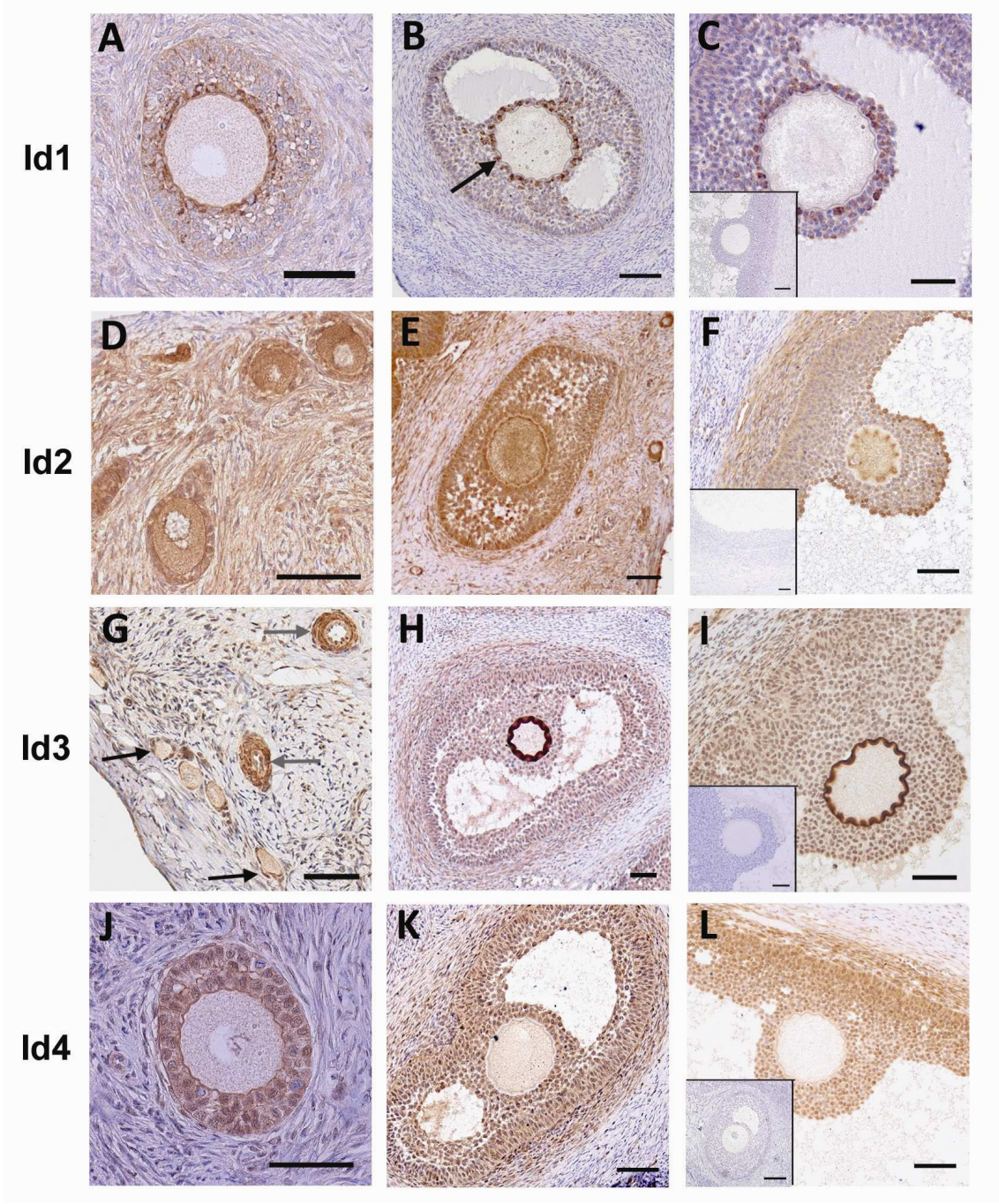

Figure 5.2 Id protein expression in the adult ovine ovary. Immunohistochemistry for Id1 (A-C), Id2 (D-F), Id3 (G-I) and Id4 (J-L) protein in the ovary, illustrates from left to right, staining in primordial or primary, secondary or early antral, and tertiary or late antral follicles. Arrow in B) indicates intense peri-oocytic Id1 expression. Insets in C), F), I) and L), are negative controls. Scale bars = 50µm.

5.3.3 Id immunostaining is reduced in the theca cells of atretic follicles

There was differential staining of Id proteins in the theca cells of some follicles (Fig. 5.3.A and B). Id1, Id2, Id3 and Id4 protein expression was reduced in the thecal layer of atretic follicles, by reference to activated caspase-3 immunostaining in serial sections (Fig. 5.3.C), while granulosa cell expression was maintained (Fig. 5.3.A and B). This phenomenon was encountered consistently in atretic follicles and could be quantified so that intense antibody staining in the thecal layer of healthy follicles was lost resulting in partial or absent expression of Id1, Id2, Id3 and Id4 proteins in follicles undergoing atresia ($P<0.0001$; Fig. 5.3.D-G). No significant differences for Id protein expression, or alteration in thecal cells in atresia, were observed between pre-antral and antral follicles (Fig. 5.3).

5.3.4 There is no specific peri-oocyte p-smad immunostaining in ovine follicles

The most striking feature of Id localisation in the ovine ovary was the intense staining for Id1 in the cumulus granulosa cells closest to the oocyte. We therefore hypothesised that an oocyte-derived paracrine factor may regulate the expression of Ids in the neighbouring cells. As members of the TGFβ superfamily are excellent candidates for an oocyte-secreted factor we investigated the localisation of the main TGFβ signalling pathways in the follicles. P-smad 1/5/8 proteins could be localised to the nucleus of both theca and granulosa cells (Fig. 5.4.A and B). Indeed, the punctate pattern of more intense staining (Fig. 5.4.B) suggests a specific regulated role for this pathway in such cells. However the staining was not increased or decreased in the granulosa cells next to the oocyte in any follicle examined. The alternative p-smad 2/3 pathway could also be detected in nuclei of both theca and granulosa cells of the follicle (Fig. 5.4.D and E). Although this staining was more uniform it also did not demonstrate a differential staining pattern in the granulosa cells surrounding the oocyte. Although it is clear that the cells surrounding the oocyte operate both smad 1/5/8 and 2/3 pathways the consistent immunostaining pattern did not reveal a direct relationship with the peri-oocyte Id1 expression in cumulus granulosa cells.

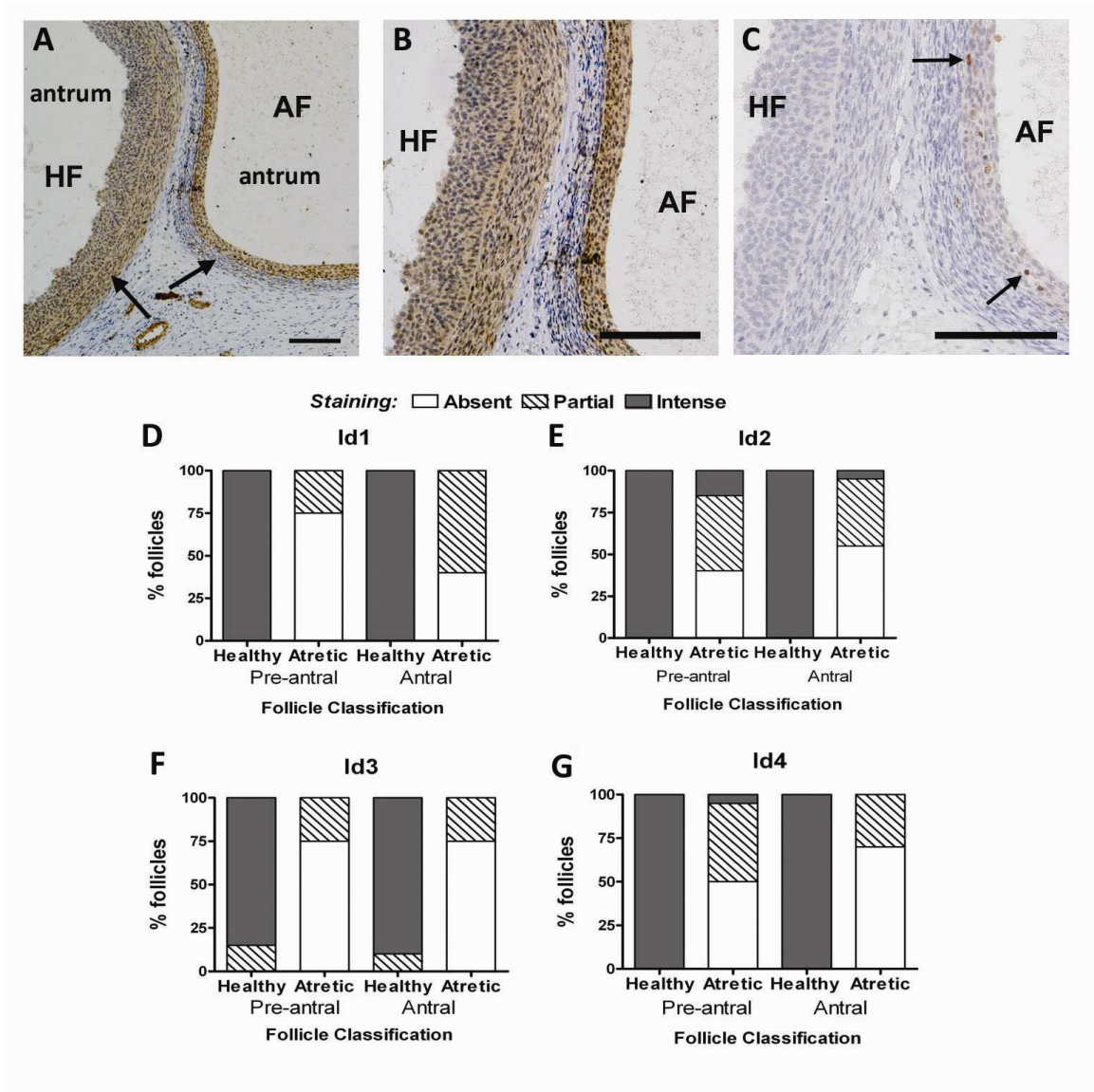

Figure 5.3 The expression of Ids in healthy and non-healthy ovine follicles. Id2 expression in an adjacent healthy follicle (HF) and an atretic follicle (AF), at low (A) and high (B) magnification, and activated caspase-3 protein expression (C) in the corresponding follicles. Arrows in A) show thecal layer and in C) depict positive caspase-3 immunoreactivity indicating apoptosis in those cells. Scale bars = 50µm. Absent, partial or intense Id1 (D), Id2 (E), Id3 (F) and Id4 (G) expression was classified in thecal cells of healthy and atretic, pre-antral and antral follicles and is illustrated for each Id. Chi-squared statistical analysis showed a significant difference between the healthy and atretic categories for Id1, Id2, Id3 and Id4, where $P<0.0001$ for each Id, for both pre-antral and antral follicle stages.

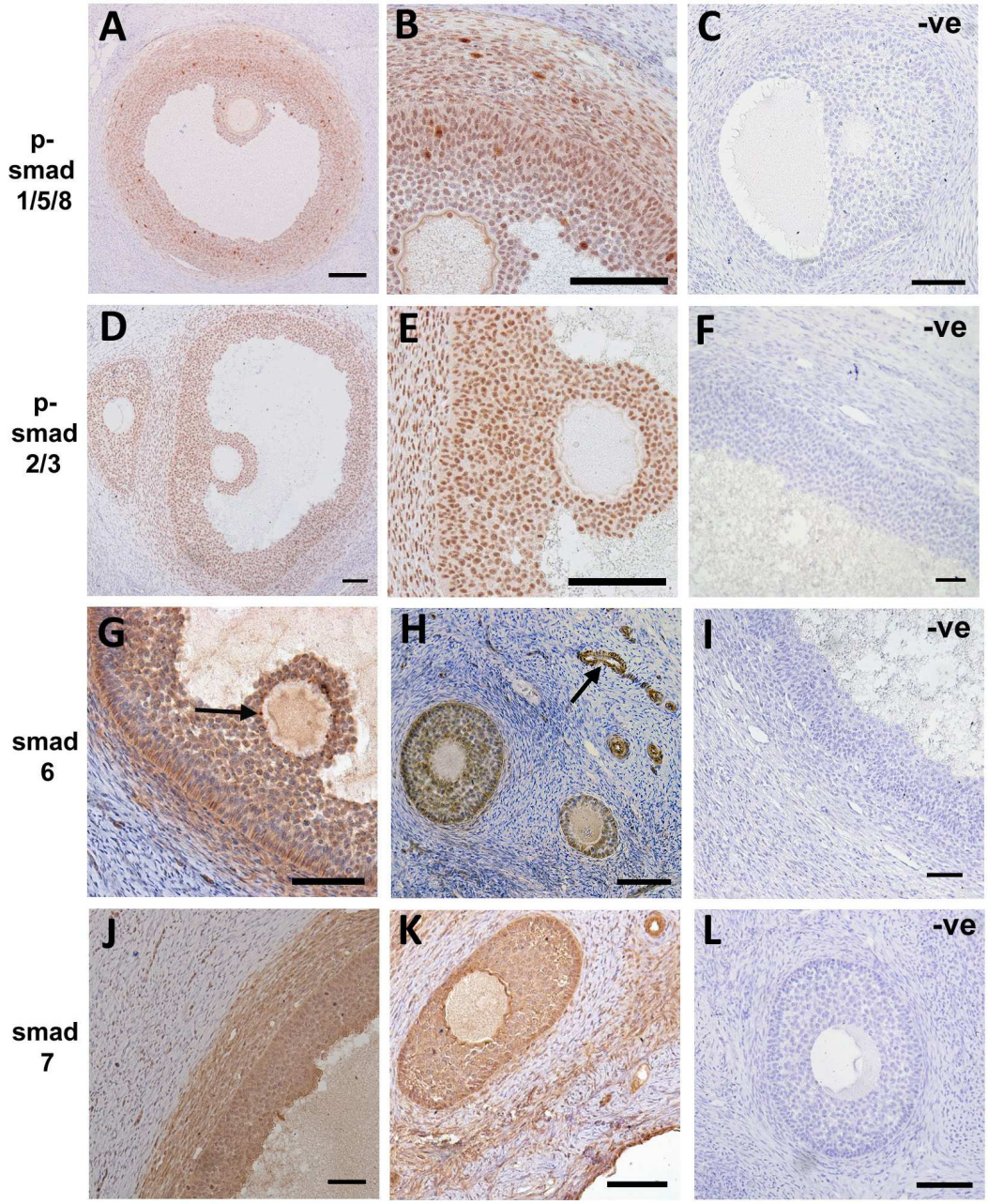

Figure 5.4 Smad protein expression in the adult ovine ovary. Immunohistochemistry for p-smad 1/5/8 (A and B), p-smad 2/3 (D and E), smad 6 (G and H) and smad 7 (J and K) in the ovary. Arrow in G) identifies smad 6 positive immunostaining in granulosa cells and in H) indicates staining in a blood vessel. Respective negative controls in C), F), I) and L) represent omission of the primary antibody. Scale bars = 50µm.

5.3.5 The peri-oocyte localisation of smad 6 in granulosa cells

Both of the inhibitory smads, smad 6 and smad 7, were found to have widespread ovarian expression (Fig. 5.4). Smad 6 revealed a similar localisation to smad 1/5/8 where staining was limited to some granulosa and thecal cells only (Fig. 5.4.G and H). However more intense nuclear staining was observed in some cumulus cells, notably around the oocyte, and also in cortical blood vessels (arrows in Fig. 5.4.G and H, respectively). Smad 7 staining was more uniform in the granulosa and thecal cells of all of the follicles examined (Fig. 5.4.J and K). To ensure the specificity of smad 6 and 7 antibody binding in the sheep, Western blotting for these proteins was also performed in ovine granulosa cell protein extracts. Specific bands were visualised for both smad 6 and 7 at the expected Mw (Fig. 5.1) confirming the expression of these proteins in this cell population and supporting the specificity of the immunohistochemistry.

The relationship of the smads to Id1 was subsequently investigated using co-immunofluorescence (Fig. 5.5). P-smad 1/5/8 did not co-localise in the cumulus layer with Id1 (Fig. 5.5.A). There was however some co-localisation between smad 6 and Id1 staining in the peri-oocyte granulosa cells (Fig. 5.5.B). This suggests that manipulation of the smad signalling system may yet have a role in the specific staining pattern of Id1 in the peri-oocyte granulosa cells.

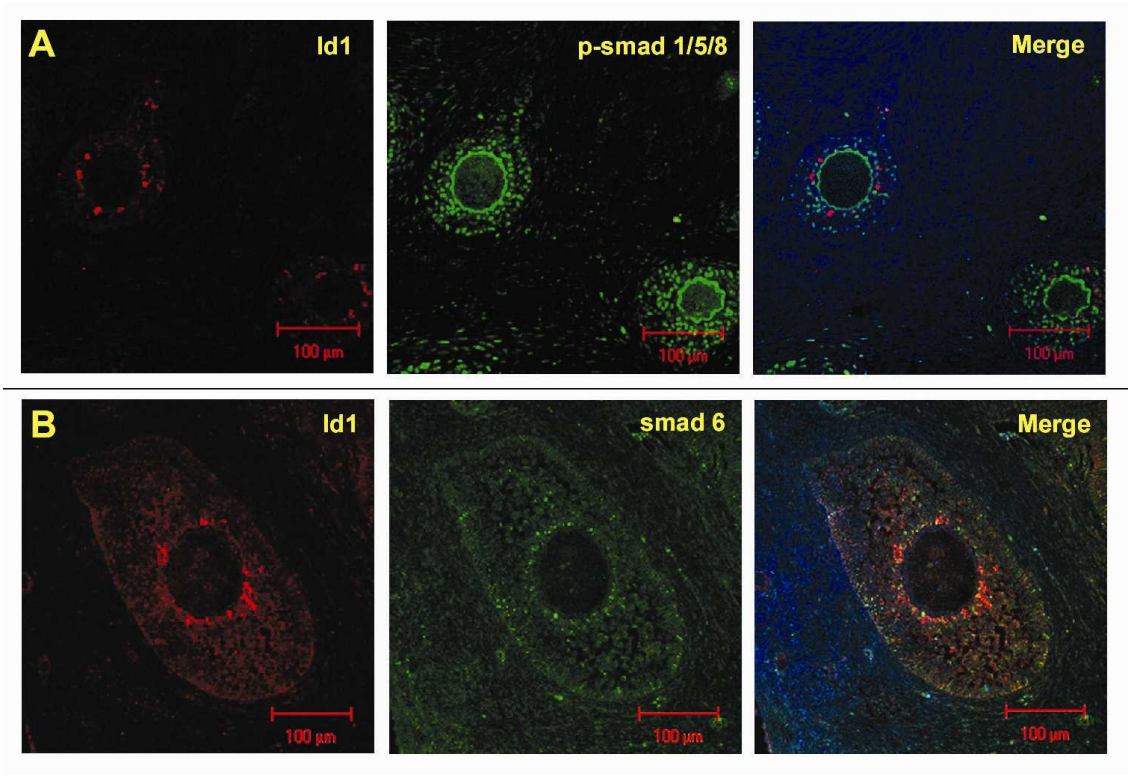

Figure 5.5 The co-localisation of Id1 protein p-smad 1/5/8 and smad 6 in the ovine ovary. Co-immunofluorescence of Id1 (red) with p-smad 1/5/8 (green) (A) and smad 6 (green) (B) in follicles of the ovary. Merged pictures include DAPI nuclear counterstain (blue). Scale bars = 100µm.

5.3.6 Activin and BMP signalling regulates *Id* gene expression in ovine granulosa cells *in vitro*

In order to investigate the effect of stimulating the smad 2/3 and smad 1/5/8 pathways on *Id* expression, ovine granulosa cells were cultured with activin A and/or BMP6 and the levels of *Id* gene mRNA were analysed by qRT-PCR. Overall ANOVA analysis revealed that *Id1* ($P<0.0001$), *Id2* ($P<0.0001$), *Id3* ($P<0.0001$) and *Id4* ($P<0.05$) were all changed by the treatments in the same pattern (Fig. 5.6). Stimulation with activin A led to the down-regulation of *Id1* and *Id3* mRNA expression whereas conversely, BMP6 treatment up-regulated these genes (Fig. 5.6.A and C, respectively; Bonferroni pair-wise analysis $P<0.001$). It was particularly notable for *Id1* expression where activin A and BMP6 treatment led to a 5-fold decrease and 7-fold increase in mRNA, respectively (Fig. 5.6A). Furthermore, treatment with activin A significantly negated the BMP6-induced up-regulation of *Id1* (Bonferroni pair-wise analysis $P<0.05$; Fig. 5.6.A).

To further explore the potential roles of TGFβ mediated regulation of Id1 expression in the cumulus cells, culture experiments were conducted where the granulosa cell population received doses of the oocyte-secreted factors BMP15 and/or GDF9 and *Id1* gene expression was measured (ANOVA $P<0.001$; Fig. 5.6.B). Similarly to the effects produced with the BMP6 cultures, BMP15 treatment also led to an increase in *Id1* transcript (Bonferroni pair-wise analysis $P<0.01$; Fig. 5.6.B). GDF9 alone did not significantly alter Id1 gene expression however in combination with BMP15 this increased 8-fold (Bonferroni pair-wise analysis $P<0.001$; Fig. 5.6.B).

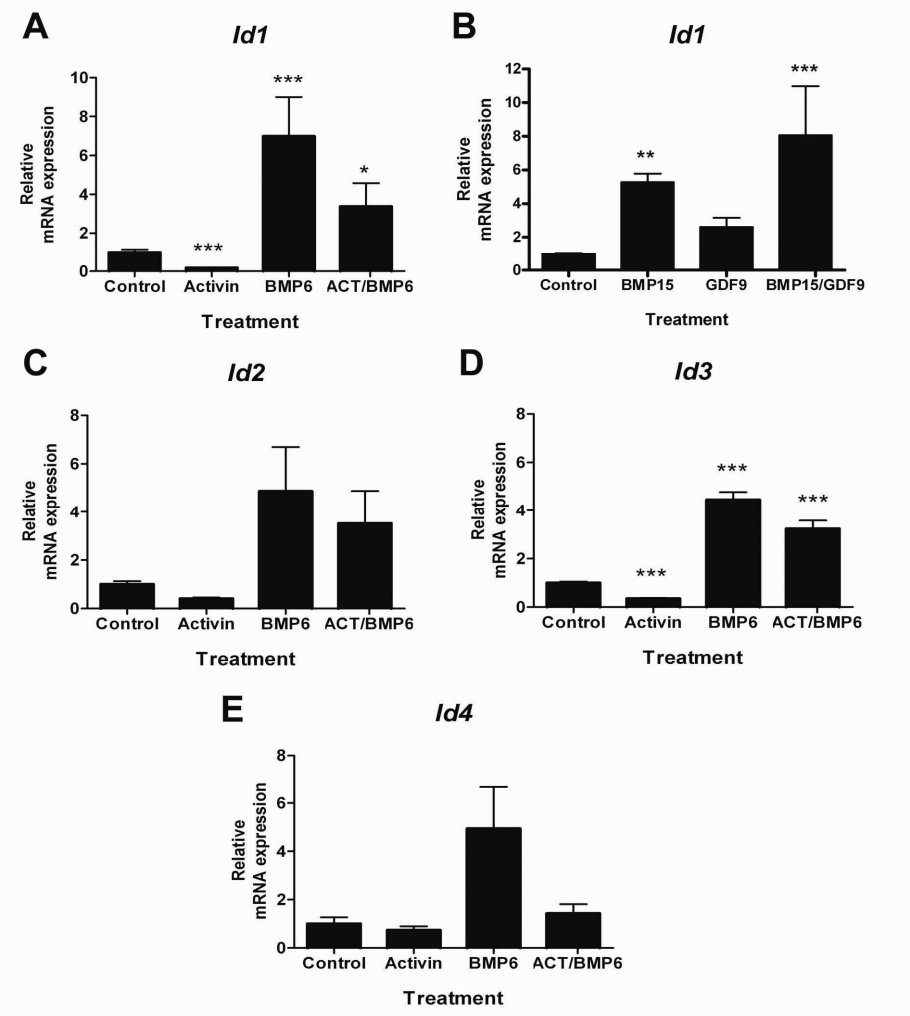

Figure 5.6 The effect of BMP signalling on Id gene expression in cultured ovine granulosa cells. Effect of activin A and/or BMP6 (A), and GDF-9 and/or BMP15 (B) on *Id1* gene expression, and activin A and/or BMP6 on *Id2* (C), *Id3* (D) and *Id4* (E) gene expression, in ovine granulosa cells cultured for 24 h, n=3; three experiments using cells from a total of ten sheep. An ANOVA analysis revealed overall significant differences for each gene; *Id1* (A; $P<0.0001$ and B; $P<0.001$), Id2 ($P<0.0001$), *Id3* ($P<0.0001$) and *Id4* ($P<0.05$) and a pair-wise comparison to the control column using the Bonferroni post test are illustrated. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

5.4 Discussion

This study describes Id protein expression across the developing mammalian ovary and provides evidence for functional roles in normal adult tissue. For the first time it has been shown that Id1, Id2, Id3 and Id4 are expressed in the granulosa and theca cells of healthy mammalian adult ovarian follicles across development. The Ids have two main functions; 1) to maintain proliferation and 2) to inhibit differentiation (553, 555). Such regulation during development ensures that the appropriate level of growth leading to patterning can occur prior to lineage commitment and the terminal differentiation of cells. The adult ovary similarly undergoes persistent tissue remodelling, resulting in the growth and differentiation of somatic follicular cells which house the oocyte. Bi-directional paracrine communication between theca cells, granulosa cells and the oocyte result in the accumulation of genetic and developmental competence for ovulation, fertilisation and subsequent embryogenesis. Id proteins are differentially expressed throughout development in many species including the mouse and *Xenopus laevis* and can have non-redundant actions as well as cell-specific roles (557, 573). We propose a role for the Id proteins in the sheep ovary that might involve transcriptional regulation critical for normal folliculogenesis that may be analogous to those observed during development.

Cumulus cell specific Id1 expression as well as the somatic cell wide Id protein expression was hypothesised to be regulated by activin and/or BMPs, present in the mammalian ovary, which are known to promote growth and differentiation in granulosa and thecal cells of various species (574-576). The steroidogenic cells of the adult ovary are fairly transient and possess similar characteristics to embryonic tissues or progenitor cells of which there are widespread reports of TGFβ-mediated Id mRNA and protein regulation (562, 563, 565). In this study it is shown that activin and BMPs can alter *Id* gene expression in ovine granulosa cells *in vitro*, and moreover, follicles have the cellular machinery for TGFβ signalling *in vivo*. This was demonstrated in ovine granulosa and thecal cells by the expression of p-smad 1/5/8 and p-smad 2/3 and the presence of smad 6 and smad 7 inhibitors indicating regulation of TGFβ signalling.

Id proteins may be regulated in ovine granulosa cells by various BMPs and activin that act in an opposing way to balance growth and differentiation appropriately. *Id1* and *Id3* expression was significantly increased following BMP6 stimulation of granulosa cells *in vitro* whereas

activin A down-regulated these genes. Therefore Id protein regulation in the sheep ovary may be a target not only of BMPs but also activin. The effect of activin on Id function is not well described however our findings are consistent with a previous study reporting a negative association with activin treatment and *Id* gene expression (564). Reports of the specific expression of BMPs in the ruminant follicle vary (574, 577, 578), however it is probable that BMP4, 6 and 7 have actions in these cells and may all regulate Id expression although this is yet to be established. We also found that treating cells with both activin A and BMP6 reduced the BMP-mediated up-regulation of *Id* genes, suggesting a competing regulatory role for these paracrine molecules.

The universally increased Id1 protein expression observed in peri-oocytic cumulus cells in follicles throughout development suggests a specific regulatory mechanism exerted by Id1 that is likely mediated by paracrine signalling from the oocyte. Although this study revealed TGFβ-mediated signalling via smad 1/5/8 and/or smad 2/3 across the whole follicle, neither pathway could be directly linked to the cumulus-specific Id1 up-regulation. P-smad 2/3 was strongly but not differentially localised in granulosa cells and while p-smad 1/5/8 expression was more limited to certain cells this was not specific to the cumulus layer. In fact, co-immunofluorescence established that in the cumulus cells Id1 and p-smad 1/5/8 were not present in the same cell and thus led to a hypothesis that smad 1/5/8 and/or smad 2/3 may indirectly regulate Id1 in these cells. Intermediary factors might include the inhibitory smads and it was observed that smad 6 displayed a more differential expression pattern with some up-regulation in the cumulus cells as well as some co-localisation with the Id1 protein. Smad 6 may repress smad signalling in some of these cells although the factor(s) regulating such an interaction are unknown. Specific oocyte-secreted factors in the sheep include TGFβ signalling molecules such as BMP6 (574) and BMP15 (GDF9b) (253) that activate smad 1/5/8 signalling in granulosa cells (579). BMP15 stimulates granulosa cell proliferation while inhibiting differentiation (580, 581) and is essential for folliculogenesis in the sheep (253).

Alternatively the lack of co-localisation observed between Id1 and p-smad 1/5/8 raises the possibility that the smad 2/3 pathway could in fact be responsible for the up-regulation of Id1 in peri-oocytic cumulus cells. Activin, an activator of smad 2/3, led to a decrease in *Id1* message in granulosa cell culture experiments. However, this effect could be cell specific since the majority of granulosa cells in these cultures are mural and the response may well be

different in cumulus granulosa cells, modulated by factor(s) secreted by the oocyte. GDF-9, an oocyte-secreted TGFβ member closely related to BMP15 (320), is an excellent candidate for triggering such a response. Its signalling pathway is less clear as it can influence both smad 1/5/8 and smad 2/3 responses (582). GDF-9 signalling has been shown to be essential for cumulus cell function where activation of smad 2/3 brings about the up-regulation of cumulus-specific genes (583). This paracrine signalling through smad 2/3 is crucial for normal cumulus cell expansion, resulting in a local milieu enabling the acquisition of oocyte developmental competence (584-586). *In vitro* GDF-9 (non-significant) and BMP15 can in fact increase *Id1* gene expression in cultured ovine granulosa cells and both ligands together enhance this response further although it is reiterated that these findings are limited to a largely mural granulosa cell population which may differ from cumulus cells. In context this latter finding is interesting since the synergy between these growth factors is known to be important for granulosa cell proliferation in the sheep (587). It is hypothesised that while the surrounding granulosa cells produce activin that might negate Id1 expression via smad 2/3, TGFβ signal from the oocyte such as BMP6, BMP15 and/or GDF-9 may lead to Id1 up-regulation in these cumulus cells.

The Id proteins were down-regulated in the thecal cells of atretic ovine follicles and not altered in granulosa cells. Follicular atresia occurs when FSH levels decrease during the follicular phase of the estrous cycle leading to apoptosis in subordinate follicles (588). The present study identifies a differential change in Id protein distribution in these follicles that may be triggered by autocrine or intracrine thecal signalling, which is potentially inhibitory and does not effect granulosa cell expression. As most changes associated with atresia are detected in the granulosa cell population these theca-specific changes are of interest. However theca-specific Id protein down-regulation in atretic follicles is merely associative with no evidence at this stage of a causal relationship.

This study is the first to carry out a comprehensive immunohistochemical examination of the Id proteins in the adult mammalian ovary. In conclusion, Id1, Id2, Id3 and Id4 are present in the ovine ovarian follicle and may be regulated by BMPs and/or activin via smad signalling. Further studies are required to establish how important the Ids are for the regulation of proliferation and differentiation of steroidogenic cells occurring during folliculogenesis and the physiological significance for fertility and reproductive diseases. Control and non-treated animals linked to the PCOS studies were used as a resource to discover new insights into

normal ovarian physiology. In Chapter 7, the ovarian phenotype of the prenatally androgenised ewe is assessed. The expression profile of the Id genes is therefore examined to determine if their expression may be changed in a PCOS-like animal model. Before this a detailed assessment of the hormonal profile and ovarian cyclicity of prenatally androgenised ewes are investigated.

Chapter 6

The effect of prenatal androgens on hormonal regulation and ovarian cyclicity

6.1 Introduction

In women with PCOS it is the disruption of the hormonal milieu that causes the characteristic features associated with PCOS that are often first noted clinically. The diagnosis of PCOS is partially based on biochemical criteria including hyperandrogenaemia that is in part secondary to enhanced pituitary secretion of the gonadotrophin LH (162). The other major clinically important feature of PCOS is irregular menstruation (oligomenorrhoea) or the complete absence of menses (amenorrhoea) due to the cessation of normal ovarian cyclicity (162).

In PCOS, perturbations to the HPO axis often involve LH hypersecretion and an increased LH pulse frequency, due in part to increased sensitivity of the pituitary to GnRH (589). Studies in the prenatally androgenised ovine fetus have identified a parallel set of features in adult ewe offspring (355, 374, 378). These endocrine anomalies occur through testosterone treatment of the mother and it is therefore possible that some of these effects are mediated by estrogen that is formed by aromatisation during placental transport and will pass into the fetus (377). Studies that use a subtractive approach by treating pregnant animals with the non-aromatisable androgen DHT have gone some way in delineating differences between the programming effects of androgens and estrogens (353, 354).

Veiga Lopez *et al.* have unequivocally identified the programming effects of alternate sex steroids using this approach (354). Through comparison of female offspring prenatally treated with TP or DHT from gestational d30-90, they revealed that in ovary-intact animals androgens affect programming of the estradiol negative feedback to the hypothalamus and pituitary that is involved in follicular selection, whereas, the positive feedback to the hypothalamus and pituitary required for the generation of the LH surge is altered by early exposure to estrogens (354). Likewise the increased LH pulse frequency and amplitude observed in prenatally androgenised sheep (299, 352) is thought to occur through increased sensitivity to GnRH mediated through androgenic programming (379).

The timing of prenatal exposure to androgens is also important. Two common models of prenatal androgenisation that exist in the sheep typically involve TP exposure from gestational d30-90 or from d60-90. These models have demonstrated differential effects on

LH surge dynamics, estradiol negative feedback and cyclicity depending on the window of *in utero* androgenisation. Generally treatment of pregnant ewes from d60-90, which is outside the window of fetal sexual differentiation, results in a more subtle reproductive phenotype (355, 369, 378) compared to those treated from d30-90 (352-354). In these studies, d60 rather than d30 was selected as a starting gestation for treatment as this avoids a severe reproductive phenotype, with marked masculinisation of the external genitalia, which is not seen in women with PCOS. In addition, this gestational period facilitates the assessment of the effects of direct administration of TP to the fetus in a parallel cohort (Chapter 3).

In women with PCOS, as well as in prenatal androgenisation models of PCOS in the sheep, non-human primate and rodent, LH secretion tends to be enhanced (295, 296, 379, 589). In addition to potential dysregulation by HPO feedback systems to either the hypothalamus or the pituitary to target LH release from the gonadotroph, there may be intrinsic changes at the level of the pituitary that exert these effects. LH may be stimulated locally in a number of ways; through reduced sensitivity to estradiol negative feedback, mediated by a reduction in pituitary ERα, by increased sensitivity to GnRH due to the up-regulation of the GnRHR, or by increased expression of the LHβ subunit itself. In fact, Mannikam *et al.* examined the expression of various pituitary genes in this regard in sheep prenatally androgenised from d30-90 a model in which increased LH secretion and pulse amplitude was present. The authors did not find significant alterations in the mRNA expression for *ERα*, *LHβ*, *FSHβ* or *GnRHR*, however the common gonadotrophin subunit *α-GSU* was up-regulated in adult sheep (379). In addition it is possible that alterations in the expression of local paracrine regulators of gonadotrophin secretion might be affected by prenatal androgenisation. These include the activins and inhibins that are positive and negative regulators of FSH secretion, respectively (93, 103, 104). In the d30-90 prenatally androgenised sheep there were trends for increases in *INHA*, *INHBB* and *follistatin*, and the activin receptor (*ACVR1*) was up-regulated in the adult pituitary, however there was no change in inhibin receptor *β-Glycan* gene expression (379). Although animal models of PCOS can inform us of the pathways involved in alterations of the hormonal milieu the molecular mechanisms of these changes have still not been fully elucidated.

The aim of this chapter is to elucidate the effect of prenatal androgens on HPO axis function in young adult sheep and to determine how the route of androgen administration or the

timing of exposure may affect the outcome. The objectives included an assessment of: A) systemic hormone concentrations, including; 1) measurement of plasma levels of androstenedione, testosterone, estradiol and progesterone (to monitor ovulation regularity) and 2) a measure of hypothalamic and pituitary function by assessment of LH pulsatility and basal LH and FSH secretion and B) pituitary gene expression for GnRH, androgen and estrogen receptivity, gonadotrophin subunit expression and local expression of the activins and inhibins.

6.2 Materials and Methods

6.2.1 Experimental animals

The adult female ewes assessed in this chapter are detailed in Table 6.1.

Study	Treatment	Sample number (n)
Adult		
Fetal Treatment	Injection at d62 and 72	C = 4, TP 20mg = 8
	Injection at d62 and 82	C = 4, TP 1mg = 5, 4mg = 5, 20mg = 7
Maternal Treatment	Treatment from d62-102	C = 5, TP = 9

Table 6.1 The experimental cohorts for the study of hormonal regulation and ovarian cyclicity, treatment regime and corresponding sample numbers.

6.2.2 Hormonal analysis

Progesterone concentration was measured in plasma obtained from weekly blood samples taken over 4 weeks from ~8.5-9.5 months of age (during late January and early February) by RIA as described in section 2.5.2 and specific assay reagents and CVs are detailed in Table 6.2. The protocol specific to the progesterone RIA and reagent concentrations are detailed in Appendix A. Progesterone values greater than 1ng/ml were considered large enough to imply probable ovulation with reference to previous studies investigating hormone secretion during the ovine estrous cycle (72, 380, 590, 591).

LH was measured in peripheral venous plasma from serial samples taken during the course of a 6 h period in adults at ~ 10-10.5 months old, in early March. Estrous cycles were synchronised prior to this assessment with an initial injection of 125mg prostaglandin estrumate (i.m.; Schering Plough Animal Health, Welwyn Garden City, UK) to terminate the luteal phase and align cycles, before a second prostaglandin injection 24 hours before the day of sampling to ensure that all animals were in the follicular phase. Animals were cannulised in the jugular vein following administration of local anaesthetic, and housed in crates with a supply of fresh water and hay during the experimental period. Sampling commenced at

10.00h and blood (1ml) was drawn every 10 min until 16.00h. Heparinised blood samples were spun at 3000 rpm for 15 min at 4°C and the plasma collected and stored at -20°C until further use. RIA was employed to measure LH at each of the time points (37 in total) as described in section 2.5.2 and reagent details and CVs listed in Table 6.2. Specific LH RIA protocol details are detailed in Appendix A. Pulses were defined if the LH concentration was >1ng/ml and were approximately one third greater or more than that of the two preceding LH values in line with previously published studies in the sheep (592).

FSH was measured on the day of serial sampling, by RIA using the same protocol for LH measurements (Section 2.5.2 and Appendix A). Table 6.2 lists specific reagent details for the FSH RIA. Two samples were measured for FSH, one from the start of sampling and one at the end (6 h apart), and the average of these values calculated. Estradiol was also measured on the same day as the serial sampling for LH took place, although this steroid was measured in one sample only from each animal (the same time point was used for all the animals). Estradiol was measured in plasma using a commercially available RIA kit of which the details and CVs are listed in Table 6.2. These samples were serum extracted prior to RIA (Section 2.5.1). Specific details for RIA measurement of estradiol are detailed in Appendix A. Testosterone and androstenedione concentrations were measured in peripheral blood samples at approximately 10 months of age. These hormones were measured by RIA following extraction of serum from the sample as described in section 2.5.1. Reagent details and CVs are listed in Table 6.2 and specific assay protocols are documented in Appendix A.

Hormone	Primary antibody	Tracer (^{125}I labelled)	Standards	Intra and Inter Assay CVs (%)
Andro-stenedione	Rabbit anti-Androstenedione (07-109216; MP Biomedicals, Illkirch, France)	Andro-stenedione (07-109226)	Andro-stenedione (A9630)	4, 7.5
Testosterone	Rabbit anti- Testosterone (R3S07-259; AMS Biotechnology, Oxfordshire, UK)	Testosterone (07-189126)	Testosterone (T1268)	4, 8
Estradiol	MAIA Oestradiol Kit (37001; Inverness Medical UK, Cheshire, UK)		Estradiol (kit)	6, 9
Progesterone	Rabbit anti-11α-Hydroxyprogesterone (PROG-#51C ; Pantex, CA, USA)	Progesterone (07-170126)	Progesterone (P9776)	8, 10
LH	In house R29*	Reichart LER-1056-C2-I125 (In-house)	LH NIAMDD-s23	6, 9
FSH	NIDDK-anti-oFSH-I (AFP-C5288113; CA, USA)	NIAMDD oFSH-I (In-house)	NIDDK-RP2 oFSH	7, 11

Table 6.2 The hormones measured in sheep peripheral plasma by RIA. A list of primary antibodies, tracer and standards employed for each RIA. Unless otherwise stated radiolabelled tracer was supplied by MP Biochemicals, Illkirch, France and the standards were from Sigma. *This in-house assay is described in McNeilly *et al*. (505).

6.2.3 Pituitary gene expression analysis

The expression of a range of genes was investigated in the adult sheep pituitary. The methodology used for these experiments are as described in Section 2.8 of the Materials and Methods chapter, which details RNA extraction, cDNA synthesis, primer validation and SYBR Green qRT-PCR protocols that were utilised for assessment of this tissue. The primer details for each gene assessed are listed in Table 6.3.

Gene (Accession No.)	Forward Sequence	Reverse Sequence	Product Size (bp)
INHBA (NM_174363)	GAAGAGACCCGATGTCACCCAGC	TGTCGTCCTCTATCTCCACGTACCCG	113
INHBB (NM_176852)	ATGGCCTGGCCTCCTCCCG	CTTCAGGTAGAGCCACAGGCTGGC	101
INHA (NM_174094)	GAGCCCGAGGACCAAGATGTCTCC	CCTCAGCCTCTCCAGCATCTGGC	91
B-Glycan (NM_214272)	AATGGCACGCACTTCATTTT	GTCCCCTGGAAATCCGTTAT	186
AR (XM_001253942)	GCCCATCTTTCTGAATGTCC	CAAACACCATAAGCCCCATC	233
ERα (NM_001001443)	GAATCTGCCAAGGAGACTCG	CCTGACAGCTCTTCCTCCTG	187
LHβ (NM_001009380)	TCACTTTCACCACCAGCATC	AAGGAGACCATTGGGTCCAC	166
FSHβ (NM_001009798)	TGTTGCTGGAGAGCAATCTG	AGTCTGCATGGTGAGCACAG	238
GnRHR (NM_001009397)	GCAGTGAAAAGCAACAGCAA	GGCAGCTGAAGGTGAAAAAG	214
GAPDH (NM_001034034)	GGCGTGAACCACGAGAAGTATAA	AAGCAGGGATGATGTTCTGG	229

Table 6.3 Adult pituitary gene analysis using SYBR Green qRT-PCR. Gene names and accession numbers, forward and reverse primer sequences (5'-3') and PCR product sizes are listed.

6.2.3.1 Statistical analyses

In this chapter, where the differences between two means were assessed, including the qRT-PCR pituitary gene expression experiments and hormonal analysis, data were statistically analysed using a student's unpaired t-test assuming equal variances. In the instances where data were not normally distributed (unequal variances) a non-parametric Mann Whitney test was alternatively employed. For the d62/82 experimental cohort in which there were numerous treatment groups, statisitcal analyses were conducted so that each TP-treated group (shaded bar) was compared to the control group (white bar). The values are presented as the mean ± sem. Data collected from proportional assessment were subjected to a chi ($\chi 2$) squared test. These values were presented as a percentage. P values of $P<0.05$ were considered statistically significant.

6.3 Results

6.3.1 Peripheral levels of estradiol, androstenedione and testosterone are not altered by prenatal androgenisation in young adults

Peripheral estradiol concentration was measured in blood collected on the same day as the pulse bleed for LH measurement. Accordingly animals were in the follicular phase of the estrous cycle as described. Estradiol, predominantly secreted by the ovary, was not changed as a result of prenatal androgenisation in maternal (Fig. 6.1.A) or fetal (Fig. 6.1.B and C) treatment groups. Androstenedione and testosterone which can be produced by both the ovary and also the adrenal gland were also not altered by prenatal androgens. There was no difference in secretion of androstenedione or testosterone in maternally (Fig. 6.1.D and G, respectively) or fetally (d62/72; Fig. 6.1.E and H, respectively) treated offspring. There appeared to be an increase in both androstenedione and testosterone in animals fetally exposed to 4mg TP (Fig. 6.1.F and I, respectively), although this did not reach statistical significance for either hormone.

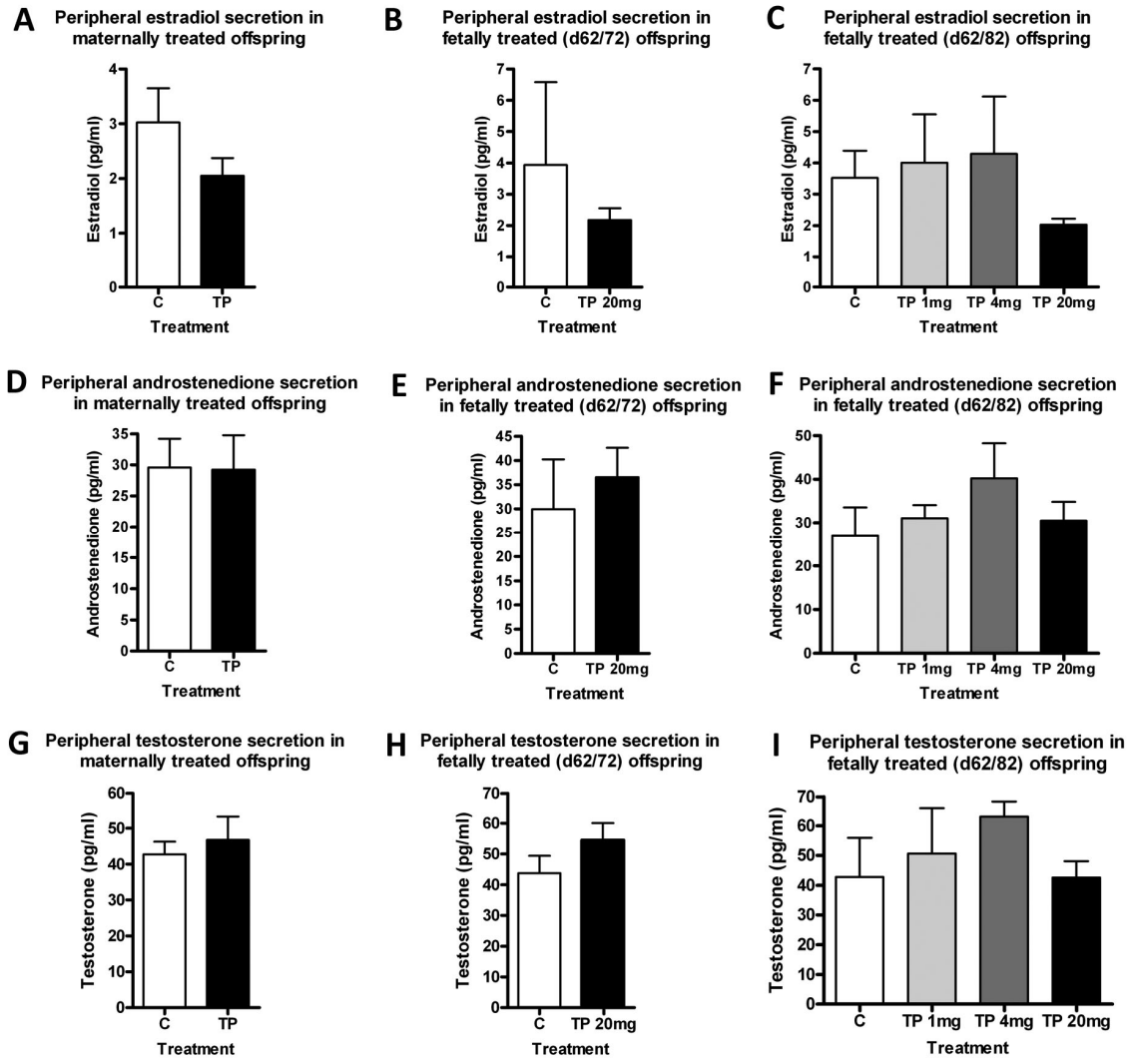

Figure 6.1 The plasma hormonal profile of young adult prenatally androgenised offspring. The effect of prenatal androgenisation in determining circulating estradiol (A-C), androstenedione (D-F) and testosterone (G-I) in young adults in maternally and fetally d62/72 and d62/82 treated cohorts, respectively.

6.3.2 Estrous cycles in the first breeding season are not universally present and HPO activation is variable regardless of treatment regime

To determine whether animals were ovulating, progesterone, which would be elevated during the luteal phase, was measured. Luteal phases, as a measure of estrous cyclicity, were classified as absent, intermittent or regular based on progesterone secretion measured over a 4 week period at ~8.5-9.5 months of age. The majority of young adult animals during this first breeding season displayed at least one high progesterone measurement (>1ng/ml) to indicate that ovulation had occurred. Two large progesterone values measured over 2-3 weeks suggested that estrous cycling was regular in those individuals; however, this was observed in fewer animals. In some animals ovulation had not occurred during the sampling period. However, prenatal androgenisation was not found to affect the number of luteal progesterone peaks in young adult animals in the first breeding season. There was no significant difference in the number of animals falling into each category following maternal (Fig. 6.2.A) or fetal (Fig. 6.2.B and C) TP exposure. There was also no overall effect of treatment strategy when control groups were compared (Fig. 6.2.D).

In addition to investigating the estrous cycle regularity in young adults, the degree of hypothalamic pituitary axis activation during the follicular phase was also assessed. Serial blood sampling over a 6 h period was performed in animals that were ~10.5 months old and LH subsequently measured to assess the frequency and amplitude of LH peaks corresponding to the follicular phase of the cycle. Like the ovulation rate amongst animals, LH pulsatile secretion was variable during this first breeding season, being present in some animals (Fig. 6.3) and absent in others (Fig. 6.4). In those animals that LH peaks were present, pulsatile secretion occurred every 2-3 hours with peak amplitudes ranging from 2-4ng/ml (Fig. 6.3). Prenatal control or TP treatment did not bear any relationship to the presence or absence of LH pulses in young adult sheep (Fig. 6.3 and 6.4, respectively, and Table 6.4). Furthermore, the number of animals with LH pulses did not necessarily equate to prostagenic cycling and often animals that displayed LH pulsatility were not previously cycling. However, these measurements were taken at different times with the measurement of LH pulsatility occurring towards the end of the breeding season.

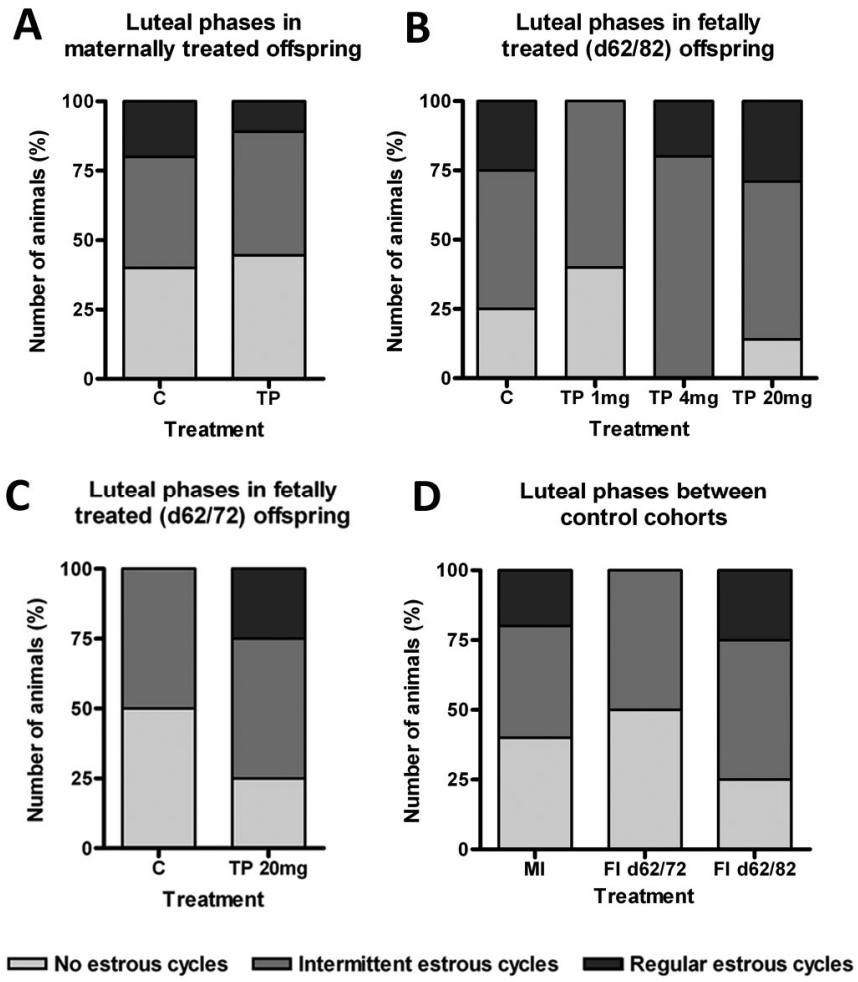

Figure 6.2 Estrous cyclicity in prenatally androgenised ewes in the first breeding season. Estrous cyclicity was determined by a peak in progesterone secretion, denoting the presence of a luteal phase, from weekly samples collected over a 4 week period during January and February in maternal (A), fetal d62/82 (B) and fetal d62/72 (C) prenatally treated cohorts. D) Compares control groups from each cohort. No estrous cycles = no discernable luteal phases. Intermittent estrous cycles = at least one luteal phase detected. Regular estrous cycles = more than one luteal phase occurring over measuremet period.

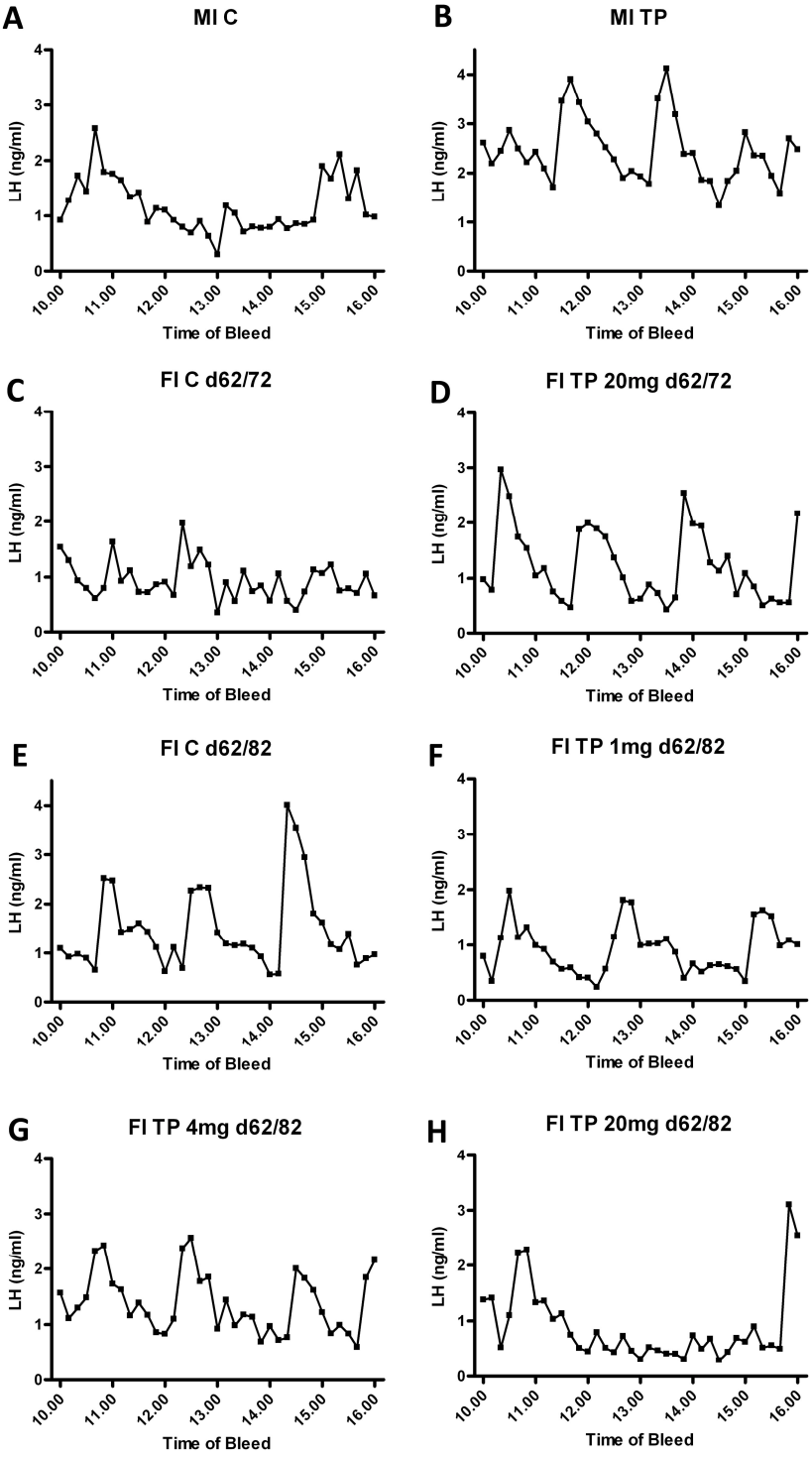

Figure 6.3 The presence of LH pulses during the first breeding season. Pulsatile LH secretion was measured over a 6 h period in early March. Illustrated are representative LH profiles from individual animals in the maternal C (A) and TP (B) cohort, the fetal d62/72 C (C) and TP 20mg (D) cohort and the fetal d62/82 C (E), TP 1mg (F), TP 4mg (G) and TP 20mg (H) cohort.

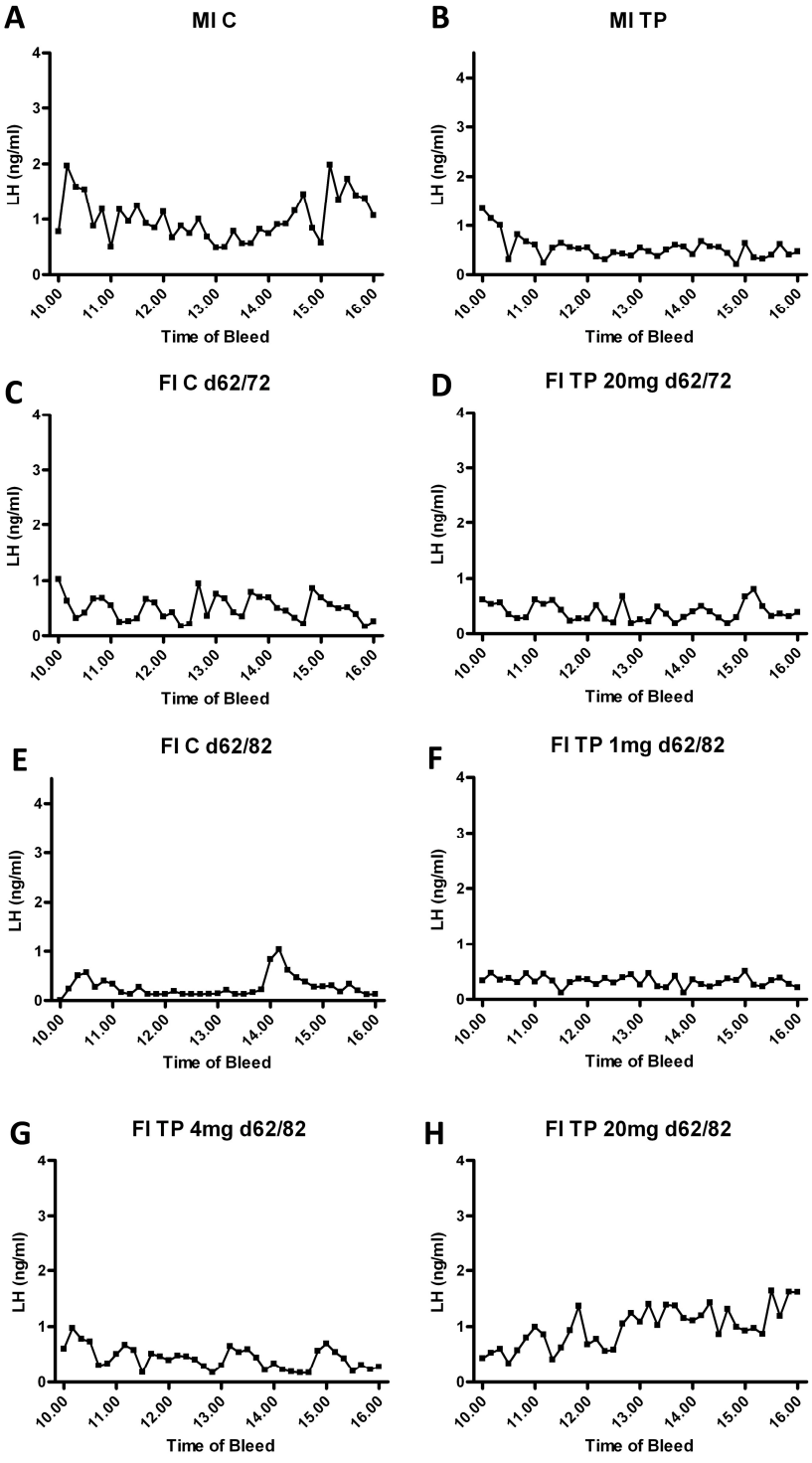

Figure 6.4 The absence of LH pulses during the first breeding season. In some animals pulsatile LH secretion did not occur when measured over a 6 h period in early March. Illustrated are representative LH profiles from individual animals in the maternal C (A) and TP (B) cohort, the fetal d62/72 C (C) and TP 20mg (D) cohort and the fetal d62/82 C (E), TP 1mg (F), TP 4mg (G) and TP 20mg (H) cohort.

	Prostagenic Cycles			LH Pulses	
Treatment	None	Intermittent	Regular	Absent	Present
MI C	2	2	1	2	3
MI TP	4	4	1	5	4
FI d62/72 C	2	2	0	3	1
FI d62/72 TP 20mg	2	4	2	3	5
FI d62/82 C	1	2	1	1	3
FI d62/82 TP 1mg	2	3	0	3	2
FI d62/82 TP 4mg	0	4	1	2	3
FI d62/82 TP 20mg	1	4	2	6	1

Table 6.4 The number of animals displaying estrous cycles and LH pulsatility during the first breeding season. Prostagenic cycling was measured from January to February whereas LH pulsatility was measured in March.

6.3.3 Prenatal androgenisation may have subtle effects on gonadotrophin secretion and pituitary function

Basal LH and FSH levels were also compared in the prenatally androgenised ewe. There was a lot of individual variation present between animals making it difficult to interpret nevertheless while there was no change in basal LH between the maternal (Fig. 6.5.A) and fetal d62/72 (Fig. 6.5B) treated cohorts, there was a trend for lower LH levels in the fetal d62/82 TP 1mg and 4mg groups and significantly less circulating LH was present in the TP 20 mg group ($P<0.05$; Fig. 6.5.C). Likewise, basal FSH levels were not altered after maternal treatment (Fig. 6.5.D) but fetal treatment at d62/72 (Fig. 6.5.E) and at d62/82 (Fig. 6.5.F) did result in lower FSH levels compared to controls, although these were non-significant trends. The LH to FSH ratio was therefore not altered after maternal androgenisation (Fig. 6.5.G) however it was non-significantly increased in the fetal d62/72 TP-treated group (Fig. 6.5.H) but not changed following fetal d62/82 treatment (Fig. 6.5.I).

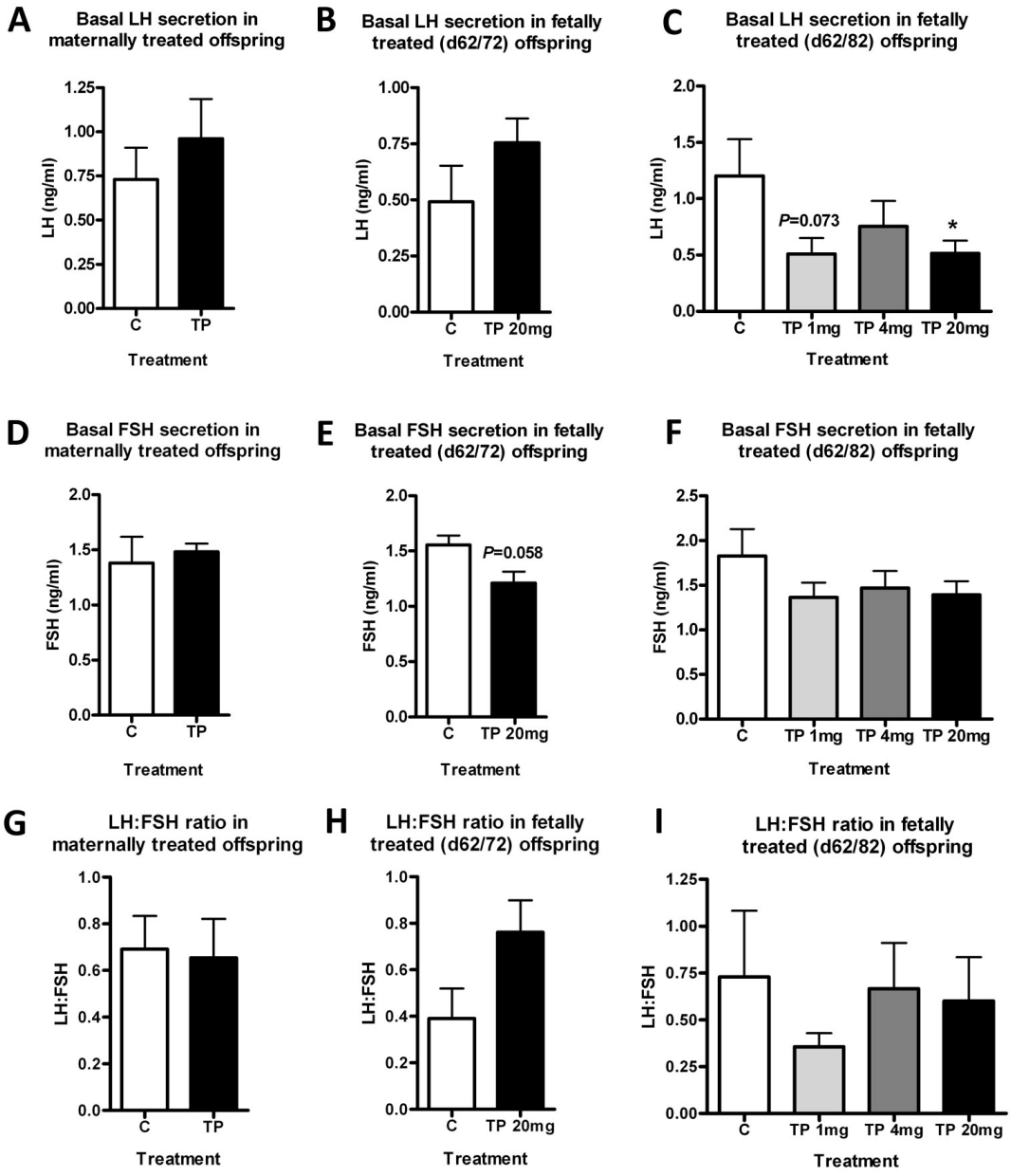

Figure 6.5 Basal LH and FSH concentrations and the LH to FSH ratio in the prenatally androgenised ewe. Basal LH (A-C), basal FSH (D-F) and LH:FSH (G-I) in maternally, fetal d62/72 and fetal d62/82 TP-exposed ewes, respectively. * $P<0.05$.

Since ewes presented varying degrees of cyclicity the levels of basal LH and FSH were compared with the presence of prostagenic cycles and LH pulsatility. Unsurprisingly the presence of intermittent and regular estrous cycles and LH pulsatility was associated with higher levels of basal LH (Fig. 6.6.A). However this relationship was not identified for FSH, with no correlation existing between FSH concentration and the presence or absence of cycles nor GnRH pulsatility (Fig. 6.6.B).

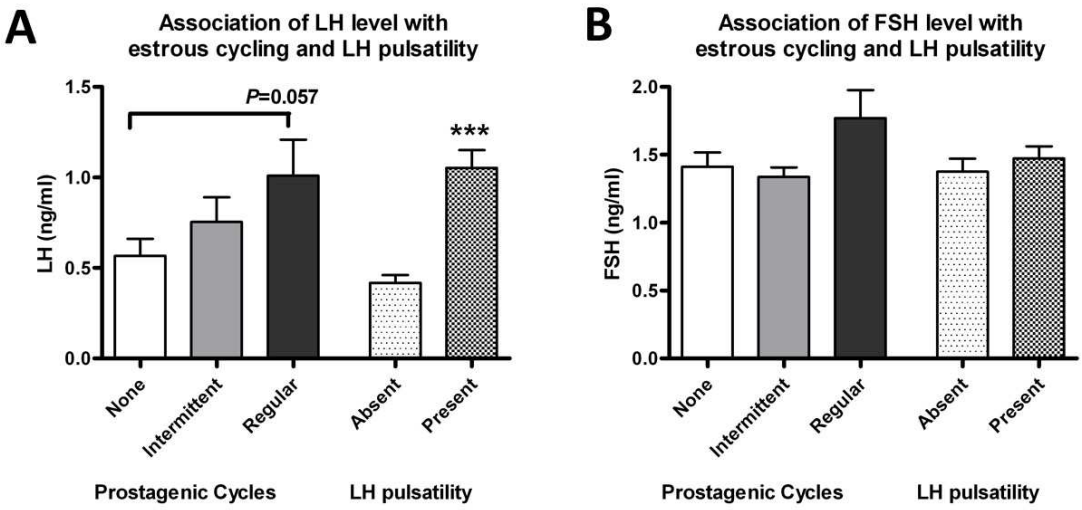

Figure 6.6 The association of LH and FSH levels with the presence of estrous cycles and LH pulsatility. All animals from each treatment cohort were grouped together as treatment regime was not shown to affect cyclicity or LH pulsatility. *** $P<0.01$. Comparison of LH concentrations in ewes that were regularly ovulating (dark shade; A) with no ovulations (white bar; A) did not reach statistical significance.

Gene expression analysis was performed in the adult pituitary to determine the effect of prenatal androgens on the receptivity to the steroid hormones androgen and estrogen and also the hypothalamic hormone GnRH. The mRNA expression of *AR* was not changed by prenatal androgenisation (Fig. 6.7.A-C) nor was the expression of *ERα* (Fig. 6.7.D-F), however there was a downward trend in *ERα* expression following fetal d62/82 exposure but this was not significant due to the variation present in the controls (Fig. 6.7.F). The expression of *GnRHR* was not altered following either maternal androgenisation (Fig. 6.7.G) or fetal d62/72 TP (Fig. 6.7.H), however pituitary *GnRHR* was down-regulated in animals that were fetally treated at d62 and d82 with 1mg TP ($P<0.05$) and 4mg and 20mg TP (non-

significant; Fig. 6.8.I). This loss of *GnRHR* mirrors the decline in LH and FSH hormone measured in the same cohorts (Fig. 6.5.C and F, respectively).

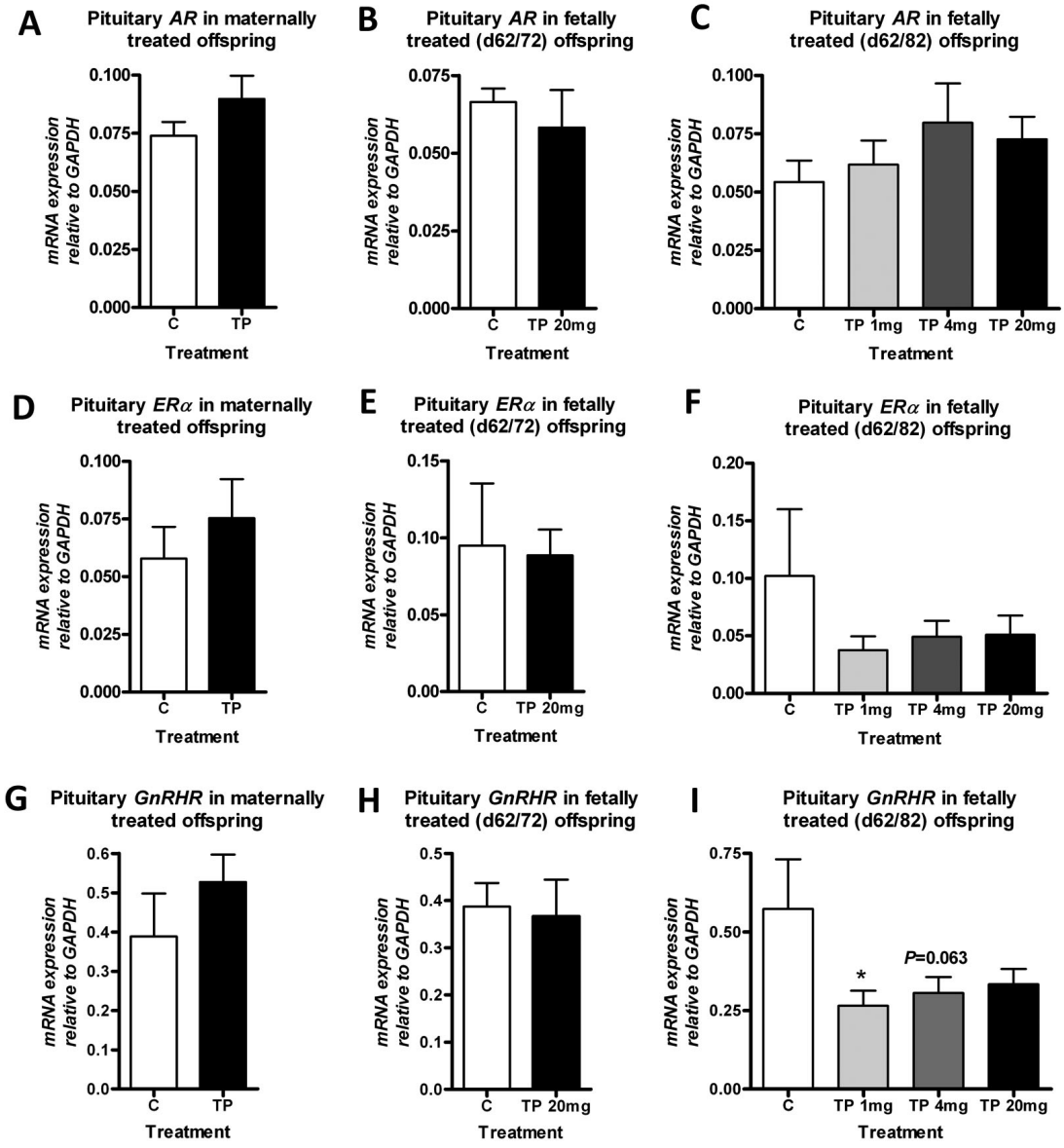

Figure 6.7 The hormonal receptor gene expression in the adult pituitary. The effect of prenatal androgenisation on gene expression in the pituitary was determined by qRT-PCR. In maternally and fetally d62/72 and d62/82 treated animals, respectively, *AR* (A-C) and *ERα* (D-F) and *GnRHR* (G-I) gene expression was assessed. * $P<0.05$.

To establish whether intrinsic changes could take place in the pituitary to govern the availability of LH or FSH, the specific subunit gene expression for these subunits was assessed. Indirect maternal TP expression resulted in an up-regulation of *LHβ* subunit expression in the adult pituitary ($P<0.05$; Fig. 6.8.A), although *FSHβ* subunit expression was unaffected (Fig. 6.8.D). Direct fetal TP treatment had no effect on *LHβ* (Fig. 6.8.B and C) or *FSHβ* (Fig. 6.8.E and F) subunit gene expression.

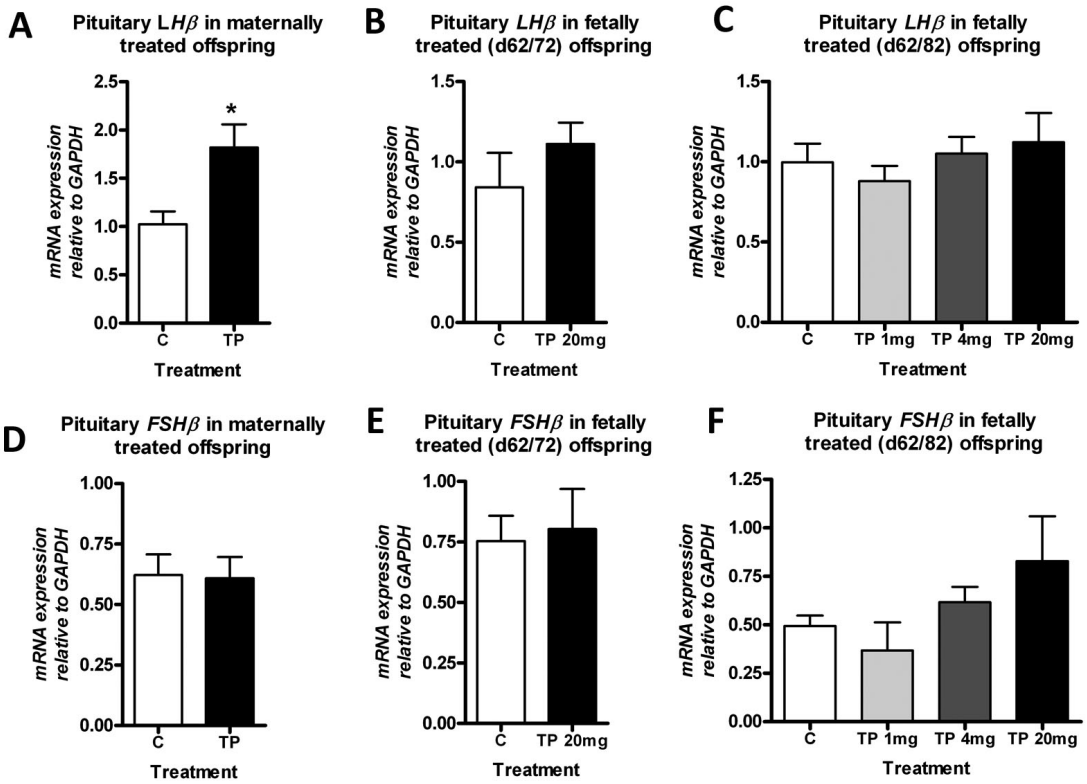

Figure 6.8 Gonadotrophin subunit gene expression in the adult pituitary. The effect of prenatal androgenisation on gene expression in the pituitary was determined by qRT-PCR. In maternally and fetally d62/72 and d62/82 treated animals, respectively, *LHβ* (A-C) and *FSHβ* (D-F) gene expression was assessed. * $P<0.05$.

The pituitary expression of the inhibin subunits, *INHA*, *INHBA* and *INHBB* were not altered by maternal or fetal prenatal androgenisation (Fig. 6.9.A-I). The expression of the inhibin receptor, *β-Glycan*, in the pituitary was also not affected by these treatments.

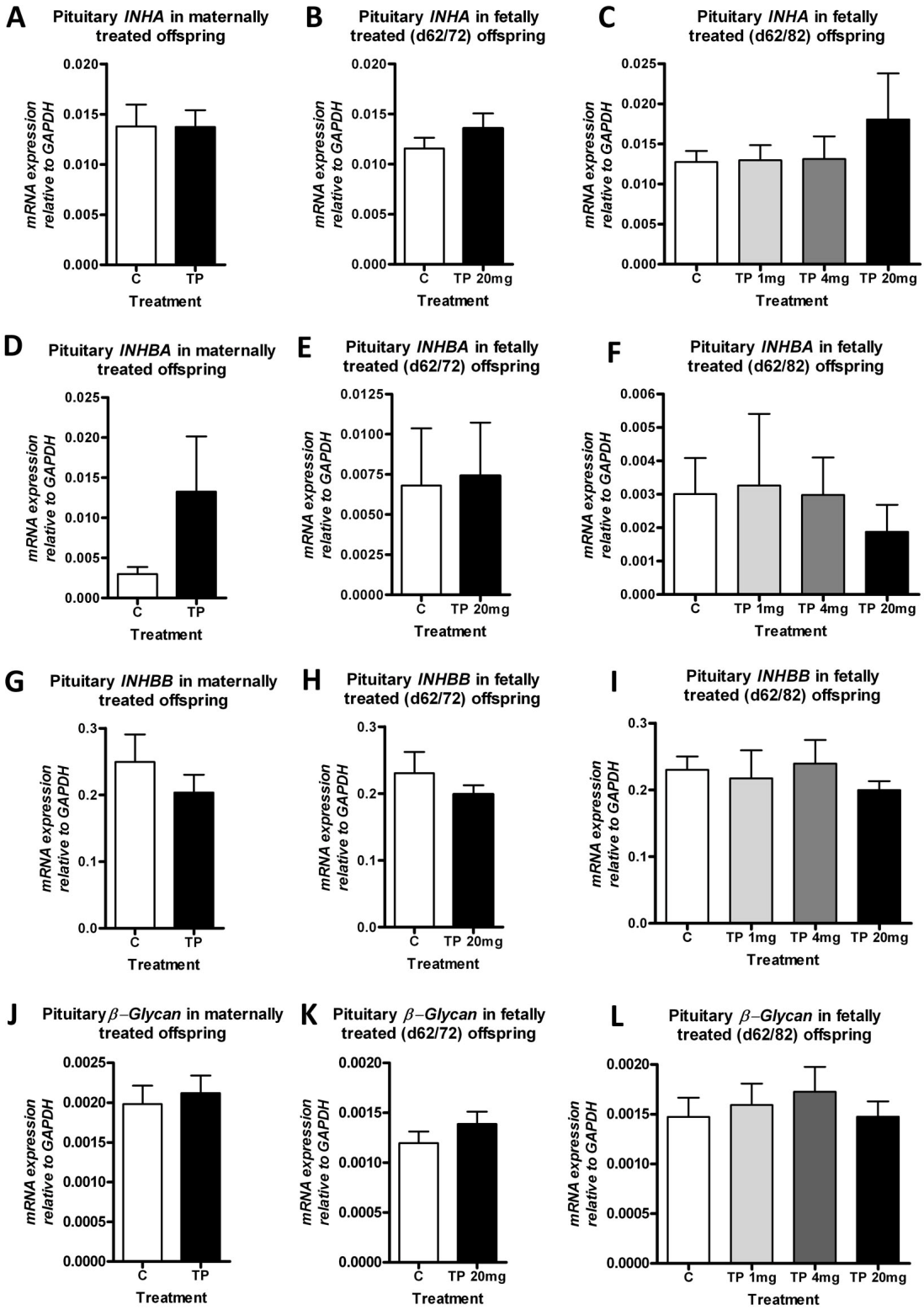

Figure 6.9 The inhibin subunit and receptor gene expression in the adult pituitary. The effect of prenatal androgenisation on gene expression in the pituitary was determined by qRT-PCR. In maternally and fetally d62/72 and d62/82 treated animals, respectively, *INHA* (A-C), *INHBA* (D-F), *INHBB* (G-I) and *β-Glycan* (J-L) gene expression was assessed.

6.4 Discussion

In this study, not all animals in either control or treated cohorts were cycling, as determined by prostagenic increases over a 4 week period during the first breeding season (January – February). In addition, only some animals exhibited pulsatile LH secretion which was measured at the end of the breeding season (March). The presence of pulsatility did not necessarily correlate with the presence of intermittent or regular cycles. In fact in the fetally injected d62/82 TP 20mg group only one animal had discernable LH pulses and it was the same individual that did not appear to be cycling at all in reference to progesterone concentrations. Conversely the other animals in this group were cycling but lacked LH pulsatility. When all of the animals from all treatment cohorts were grouped together ~75% of them were intermittently or regularly ovulating, however, only ~50% had signs of LH pulsatility. The lag time of one month between these experiments has likely caused this discrepancy and measurement of LH at this time was towards the end of the breeding season at which point some animals may already have entered hypothalamic suppression of gonadotrophin secretion and therefore estrous cycling. Also the fact that not all animals were fully cycling throughout the first breeding season suggests that some young adults were still immature and had not fully switched on their reproductive axis at this time.

Despite clear prenatal androgenisation of an ovine fetus from gestational d62-102, there were no obvious endocrine anomalies by the first breeding season in adult offspring. In line with previous reports investigating the programming effect of prenatal androgens during this mid-gestational period, this study reveals an overall more subtle effect on adult endocrine function than other studies in which prenatal exposure to androgens has occurred over a longer and crucially, earlier, time in fetal development, coinciding with sexual differentiation (369). Androgen exposure of a female ovine fetus from d30-90 effects the timing of tonic LH initiation (353-355) and results in acyclicity in the second breeding season (355), LH hypersecretion and an increased GnRH (LH) pulse frequency (352). These animals exhibit a masculinised phenotype and are born with male external genitalia, including a penis and scrotum (370). In female ovine fetuses exposed to androgens after this period of neuroendocrine sexual differentiation, between d60 and d90, the effects on adult hormonal and ovarian function are less severe (369).

Birch *et al.* revealed that the timing of puberty is still advanced in d60-90 animals but rather than complete anovulation, sheep have intermittent cycles in their second breeding season (355). Studies assessing estradiol feedback and cyclicity over successive breeding seasons found that in the first breeding season there was no effect of TP from d30-90 or d60-90 on the timing, number and duration of ovulatory cycles, however, estradiol positive feedback was affected in both cohorts illustrated by a delay in the LH surge that was even longer in the d30-90 group (378). In the same study, the magnitude of the LH surge in the d60-90 group was not affected however it was dampened in the d30-90 cohort (378). In the second breeding season d60-90 treated animals displayed regular estrous cycles (299) however in d30-90 treated adult offspring more than half of the animals were acyclic (379). By the third breeding season d60-90 TP exposed animals had normal cycle regularity and luteal function, however, LH pulse frequency was increased and the timing of the LH surge was still affected (299). The concentrations of testosterone in the fetal treatment cohort are supraphysiological (Chapter 3) and the effects are similarly negative when compared to the maternal treatment cohort. This confirms that programming of the hypothalamic pituitary axis is based of time and duration of exposure rather than degree of exposure.

Not all of the ewes in this study were cycling at the time of assessment, implying that the HPO axis had not fully matured in some animals. Accordingly basal LH levels were correlated with the presence of prostagenic cycles and LH pulsatility, however, basal FSH was not. Basal FSH levels were nevertheless reduced in both fetal treated cohorts as a result of prenatal androgenisation although this was a non-significant trend. Interestingly basal LH was also reduced in the fetal d62/82 TP exposure group, significant at the 20mg dose. Since there were no changes in the gene expression of either *LHβ* or *FSHβ* subunits in these cohorts, it is possible to speculate that the lowered gonadotrophin levels in this cohort could be directly related to the down-regulation of pituitary *GnRHR* in the same animals. Since GnRH is known to regulate the expression of GnRHR in the pituitary it may be that in these animals GnRH signalling is dampened or chronic low frequency GnRH may drive down-regulation of its receptor (593), although this was not assessed. Alternatively, given that there are no peripheral changes in estradiol or testosterone levels, nor pituitary *AR* or *ERα* mRNA, that may affect GnRHR expression through feedback pathways (593), the change in *GnRHR* gene expression could be an intrinsic effect of direct prenatal androgenisation in which the sensitive developmental period occurs around d80 of gestation or after.

Mannikam *et al*. also postulated that the LH hypersecretion that is a consequence of prenatal androgens from d30-90 of gestation, may be due to changes in pituitary gene expression. Similarly to our ovine model, they did not identify any alterations in the expression of *ERα*, *FSHβ* or *GnRHR* in the adult pituitary of maternally treated offspring (379). However, unlike these authors, we found that maternal androgenisation over mid-gestation did result in an up-regulation for the *LHβ* subunit. The increase in *LHβ* subunit expression may indicate an increased gonadotroph drive for synthesis and storage of this hormone that has not yet become, or is stimulated to be, hyper-secretory. Our animals, unlike the cohort in the Mannikam *et al*. study were not LH hypersecretory, however our animals were 11 months of age opposed to 22 months. It is possible that this phenotype had not developed in our cohort or, due to the later period of androgenisation, was not a programmed effect (378). Robust conclusions are however difficult to come to when not all of the animals appear to have a fully functional HPO axis. Nevertheless, we did not identify alterations in the inhibin subunit expression profile or the regulation of the inhibin receptor, β-Glycan, in agreement with the findings of Mannikam *et al*. (379).

In this chapter, the hormonal dynamics in response to prenatal androgenisation via the mother or directly to the fetus during mid-gestation were assessed. Overall, there were no changes in ovarian cyclicity, peripheral steroid hormone secretion and GnRH pulsatility as measured by repeated LH measurement, as a consequence of these treatments. It is apparent however that an assessment in the more mature animal during the second breeding season may have revealed more changes in these regards, had this been feasible. Nevertheless, there were some effects of prenatal androgenisation that were present in the pituitary, despite the fact that the HPO axis may not have been fully active at this time, and this suggests that these are intrinsic effects that occur independently of feedback signalling from the ovary. Importantly, however, in an animal model, where the fetus is exposed to androgens during mid-gestation, the endocrine system in the young adult is essentially normal. This can therefore provide a platform to assess the effects of fetal androgen programming in the ovary, and other tissues, in the absence of other variables, such as high LH concentrations, hyperandrogenism and differences in ovarian cyclicity that could influence the overall phenotype. In the following chapter, this was utilised to determine if there were intrinsic differences in the ovary in the absence of a PCOS-like hormonal milieu.

Chapter 7

The effect of prenatal androgen exposure on ovarian function and folliculogenesis

7.1 Introduction

One of the defining features of PCOS is the presence of polycystic ovaries (162, 163). Morphologically, PCO are multifollicular, containing an increased number of antral (2-8mm) follicles that are located in the periphery of the ovary of which the larger follicles (5-8mm) are growth arrested (594). There is often failure to select a dominant follicle for ovulation leading to oligomenorrhoea or amenorrhoea (163). While PCO ovaries are present in most women with PCOS, this ovarian phenotype is present in ~20% of 'normal' women of reproductive age although PCO morphology is correlated with increased androgens and increased menstrual irregularity in these women (162, 595).

In animal models, like the programming effects on the HPO axis, ovarian morphology and follicle dynamics are disrupted in prenatally androgenised offspring in a manner reminiscent of PCOS (316, 350, 427). In lambs, a polyfollicular phenotype occurs through prenatal androgenisation from d30-90 with TP, but not DHT, indicating an estrogenic mode of programming (301). Similarly PCO have been observed through early exposure to androgens in non-human primates (356) and rodents (364, 365). Disrupted folliculogenesis may occur through a number of pathways. Evidence from women with PCOS (317, 318) and prenatally androgenised sheep (332, 333) supports a role for increased primordial follicular recruitment into transitionary and primary follicle stages, with a reciprocal reduction in the primordial reserve. Moreover it appears that the level of follicular regression through atresia is diminished in both women (319) and sheep (334).

The hyperandrogenic hormonal environment in PCOS may also be of importance in producing disordered folliculogenesis: the effects of which may be additive to fetal androgenic programming. In normal-cycling adult rhesus monkeys treated with testosterone for a short period (3-10 days) the number of growing follicles was increased, there was a greater thecal cell number and enhanced granulosa cell proliferation (336). An increased androgenic environment also led to up-regulation of *AR* (340), *IGF1* (337) and *FSHR* (341) in the granulosa cells of small growing follicles of rhesus monkeys. These findings imply that signalling through the AR may play a role in enhancement of the IGF1 signalling and sensitivity to FSH, both crucial factors involved in folliculogenesis (338, 339).

A role for disordered activin, inhibin and follistatin in PCO has also been proposed. Activin and inhibin are members of the TGFβ superfamily that have opposing actions (80). They are made up of inhibin subunits encoded by the *INHA*, *INHBA* and *INHBB* genes (79) (Fig. 1.5) and activin signalling is essential for folliculogenesis (112, 113, 115, 116). Activin is proposed to act to promote granulosa cell function and proliferation, yet prevent premature luteinisation (118, 119). In the prenatally androgenised lamb, intrafollicular activin availability may be targeted through a follicular increase in follistatin (301), a glycoprotein which sequesters activin (87). Furthermore, the *INHBB* subunit also tended to be lower in these follicles suggesting a loss of local activin production (301).

As the follicle progresses through development, activin levels fall, while inhibin levels rise (124). Inhibin, in the presence of LH, enhances androgen production from human (120), rat (121) and bovine (122) thecal cells, while activin suppresses this action (120, 122). Increased ovarian androgen production is thought to contribute to the hyperandrogenism in PCOS (344, 345, 596), in conjunction with enhanced adrenal androgen secretion (415, 597). Thecal cell androstenedione is increased in women with PCOS (344) which is augmented not only by the hypersecretory LH state and increased expression of thecal *LHR* (598), but also intrinsic alterations in thecal steroidogenic gene expression (345, 598). Global enhancement of the steroidogenic pathway is found in thecal cells from women with PCOS with up-regulation of *StAR* (598), *CYP11A* and *CYP17* (345, 598) gene expression and increased enzyme activity of 17α-hydroxylase, 3β-HSD and 17β-HSD (345).

A PCO ovary is therefore characterised by disrupted folliculogenesis, altered expression of growth factor systems and enhanced ovarian androgen production. These features are additionally associated with the hormonal environment found in PCOS, including hyperandrogenism and LH hypersecretion. Whether the perturbed function in the ovary is caused by these peripheral hormonal changes, originates from early programming events, or is a combination of both, is not known. The studies outlined in Chapter 4, suggest that there are direct functional programming effects of androgens on the fetal ovary that may affect developmental signalling systems and steroidogenesis. The data presented in Chapter 6 show that mid-gestation prenatal androgen exposure does not affect peripheral hormonal concentrations, including androgens or LH, in the first breeding season. Therefore this allows for assessment of the primary effects of prenatal androgenisation on the adult ovarian phenotype without any impact from an abnormal hormonal environment.

The objectives of this chapter are to assess the impact of mid-gestation androgen exposure on the ovary by assessing: A) ovarian morphology and follicular dynamics, and B) follicle function, including 1) thecal cell androstenedione secretion and response to stimulation with LH, and 2) thecal cell gene expression related to steroidogenesis, hormone signalling and growth and differentiation, including the Id genes, identified as having a role in normal follicular function in Chapter 5.

7.2 Materials and Methods

7.2.1 Experimental animals

The adult female ewes assessed in this chapter are detailed in Table 7.1.

Study	Treatment	Sample number (n)
Lamb (ovarian morphology)		
Fetal Treatment	Injection at d62 and 72	C = 6, TP 20mg = 6
Maternal Treatment	Treatment from d62-102	C = 9, TP = 7
Adult		
Fetal Treatment	Injection at d62 and 72	C = 4, TP 20mg = 8
	Injection at d62 and 82	C = 4, TP 1mg = 5, 4mg = 5, 20mg = 7
Maternal Treatment	Treatment from d62-102	C = 5, TP = 9

Table 7.1 The experimental cohorts for the study of adult ovarian function and folliculogenesis, treatment regime and corresponding sample numbers.

7.2.2 Histology

Lamb and adult ovaries were fixed in Bouins solution for 24 h prior to processing and blocking in paraffin wax. Sections (5µm) were cut and mounted onto charged glass microscope slides. Ovarian sections were either stained for H&E or underwent immunohistochemical staining as described in Section 2.4.2 and 2.4.3.1, respectively. Antibodies and optimised working concentrations are detailed in Table 7.2.

7.2.3 Assessment of follicle number, growth and cell death

H&E stained or immunostained ovaries were assessed to calculate follicle number, including primordial, primary, pre-antral and antral follicle stages, or follicle proliferation and cell death suggesting atresia, respectively. Animals included in this assessment were maternally exposed (C = 5, TP = 9) or fetally exposed to androgens at d62 and d82 (C = 4, TP 4mg = 5).

Antigen	Antibody Clone/Source	Dilution	Secondary antibody
AR	Polyclonal Rabbit (N20; sc-816) Santa Cruz Biotechnology Inc., Santa Cruz, USA	1:100	GARB
Cleaved Caspase-3	Polyclonal Rabbit (Asp175, #9661) Cell Signalling Technology Inc., USA	1:100	GARB
Ki67	Monoclonal Mouse (NCL-Ki67-MMI), Novocastra Laboratories, Newcastle, UK	1:200	GAMB

Table 7.2 List of antibodies used for immunohistochemistry in the adult ovary. Antibody suppliers, working concentrations and appropriate secondary antibodies are listed.

7.2.3.1 Assessment of follicle number

Follicles were categorised into developmental stage according to human and ovine classification of follicle stage (23, 24). Primordial or resting follicles were classed as an oocyte surrounded by one layer of flattened granulosa cells (Fig. 7.1.A and B). Primary follicles contained an enlarged oocyte surrounded by one layer of cuboidal granulosa cells (Fig. 7.1.C). Follicles were classed as pre-antral if they contained two or more layers of granulosa cells, had formed a basement membrane and had begun to recruit thecal cells (Fig. 7.1.D). Antral follicles contained many layers of granulosa cells, surrounded by a basement membrane and outer layers of thecal cells and had formed an antral cavity containing follicular fluid (Fig. 7.1.E and F).

Follicle counting was carried out in at least 8 ovarian H&E stained sections from each individual, which were a distance of 100µm apart, to give a representative follicle count throughout the ovary. Counting was performed using a light microscope (Zeiss Standard 20, Carl Zeiss) and a manual clicker. The average number of follicles per section for each developmental stage classification was calculated for each animal and these values used for statistical analysis. The number of primordial follicles present in the ovary was not normalised to ovarian weight as primordial follicle number is finite and pre-determined. Since ovarian weight was not altered between treatment groups absolute follicle numbers per ovarian section are also described for those follicles beyond the primordial stage.

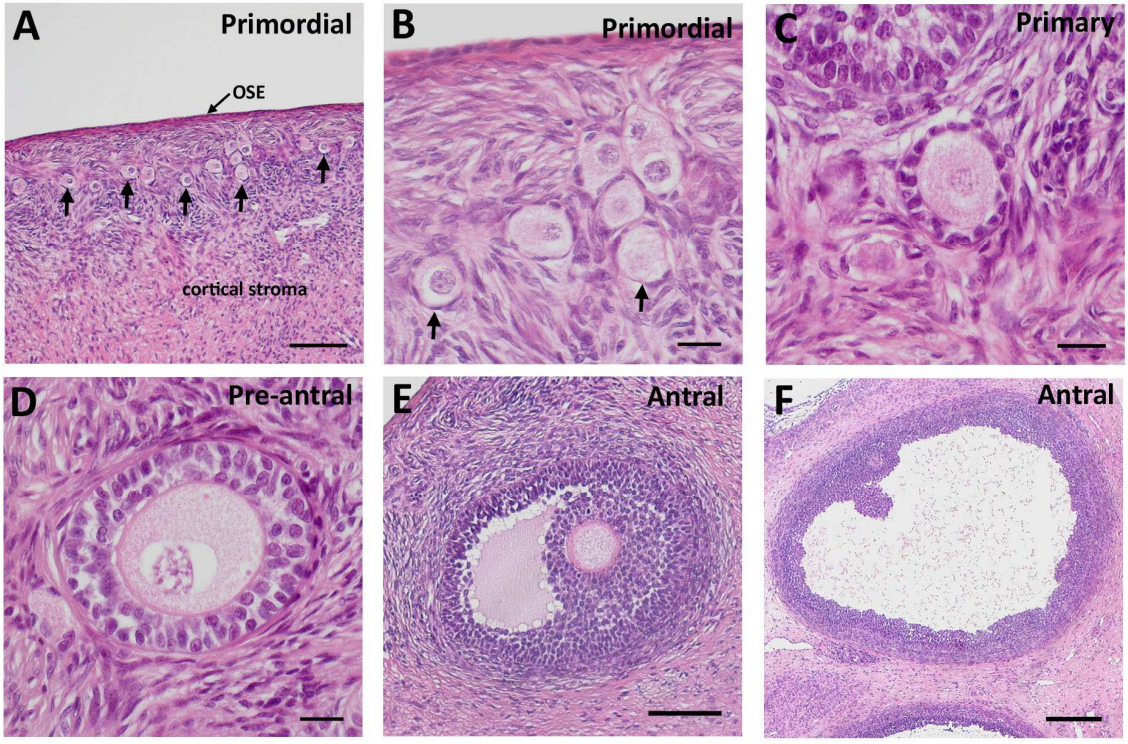

Figure 7.1 Follicle developmental classifications. H&E staining of primordial (A and B; indicated by arrows), primary (C), pre-antral (D), early antral (E) and late antral (F) ovine follicles. Scale bars represent 100µm (A, E and F) and 20µm (B-D).

7.2.3.2 Assessment of antral follicle proliferation and atresia

The same method for follicle counting was employed to quantify the number of antral follicles immuno-positive for Ki67 (a proliferative marker) and cleaved caspase-3 (a cell death marker). The average number of non-growing and growing or healthy and dying follicles per section was calculated for each animal and this proportional value used in the analysis[3].

[3] Ovarian sectioning, H&E staining, follicle counting, immunohistochemistry and histological assessment undertaken for this Chapter was carried out, in part, by Elizabeth Oliver under supervision by KH.

7.2.4 Individual follicle analysis

For these studies maternally treated (C = 5, TP = 9) and fetally treated at d62 and 82 animals (C = 4, TP 20mg = 7) were selected. Sample numbers varied between experiments depending on the number and particular size of follicles that an individual ovary contained and will be referred to in each section. Ovaries were collected at 11 months of age immediately after the animal was euthanised. One ovary from each animal was placed in a petri dish containing PBS and cut in half. Individual follicles (>1mm) were carefully dissected using scissors and fine forceps under a dissecting microscope and cleaned as described previously (72). The diameter (mm) of each individual follicle was recorded.

7.2.4.1 Collection of follicular fluid, thecal cell collection and culture

The follicular fluid from each follicle was collected and stored separately. Each follicle was transferred to an individual well containing 1ml PBS in a 24 well plate, the follicle bisected and opened up releasing the follicular fluid, and the granulosa cells scraped out. The thecal shell was removed and the PBS containing follicular fluid and granulosa cells were separated by centrifugation at 400g for 10 min at room temperature. The fluid was collected and stored at -20°C and the granulosa cells were resuspended in 100µl RNAlater (Applied Biosystems) and stored at -20°C.

For follicles up to 2.5mm, the thecal shell was cut into two halves. For follicles >2.5mm the thecal shell was peeled from the inside of the follicle shell, and also cut into two similar size pieces. For both sizes of follicles, one half was immediately placed in 250µl RNAlater (Applied Biosystems) and stored at -20°C. The other half was cultured in 24-well plates, initially in 1ml medium 199 for 1 hour and then transferred to media containing 100ng/ml LH (NIDDK ovine LH 23) and cultured a further hour at 37°C/5% CO_2. After the culture period, the thecal shells were snap frozen and stored at -80°C and the media from both basal and LH cultures was stored at -20°C[4].

[4] Follicle dissection, measurements and thecal cell cultures were performed by Drs. Carlos Souza, Julia Young and Prof. Alan McNeilly.

7.2.4.2 Androstenedione and estradiol measurements

A RIA for androstenedione was performed in culture media collected from the thecal cell cultures (pre- and post-LH stimulation) and from follicular fluid, as described in Section 2.5.2, and the reagents required and CVs are detailed in Table 6.2, in Chapter 6. Every culture media or follicular fluid sample corresponding to follicles that were ≥3mm in diameter was included in the assay and at least 3 follicles that were <3mm were included for each animal. Since androstenedione was not in this instance measured in plasma, the serum extraction step was not necessary. One deviation from the specific protocol for this assay, described in Appendix A, was the use of 20µl follicular fluid in 180µl buffer per assay tube (the volume of culture media used remained at 100µl).

Estradiol was also assayed in same follicular fluid sample in which androstenedione was measured, using an in-house ELISA protocol. On day one, Nunc Immuno 96 MicroWell plates (Fischer Scientific Ltd.) were coated with 100µl of donkey anti-sheep IgG (1:200; 713-005-147, Jackson ImmunoResearch, Suffolk, UK) diluted in coating buffer (1 tablet carbonate–bicarbonate buffer tablet [Sigma] in 100ml de-ionised water). Plates were sealed and stored overnight at 4°C in a humidity chamber to prevent evaporation. On day two, coating buffer was discarded and the plate washed three times with 200µl wash buffer (de-ionised water + 0.1% Tween-20). Estradiol antibody (In-house ASMR32; 1:300,000, diluted in 0.1M PGBS) in a volume of 100µl was added to each well. A NSB well was included as a control, with the addition of 100µl PGBS. The plate was sealed and incubated for 3 h at room temperature. The liquid was discarded and the plate washed three times with 200µl wash buffer per well. For the standards, 100µl of standard (0.78, 1.56, 3.2, 6.25, 12.5, 25, 50, 100 pg/100µl β-estradiol; E8875, Sigma) was added to the wells in duplicate. The optimal sample dilution was determined and re-assayed if required. Sample volumes ranged from 1, 10, 20 or 50µl follicular fluid, diluted with PGBS to a final volume of 100µl. Diluted samples were added to wells in duplicate. In NSB and B0 wells, 100µl PGBS was added. The tracer (estradiol conjugated to HRP; In-house) was diluted 1:500 in PGBS and 50µl added to each well including T0 wells. The plate was sealed and incubated for 2 h at room temperature. Following this, liquid was discarded and the plate washed five times with 200µl wash buffer. Substrate was prepared consisting of 33ml 0.1M citrate phosphate buffer pH 5.0 (10.30g citric acid and 17.79g HNa_2O_4P, dissolved in 1l de-ionised water), 33µl 30% hydrogen peroxide and 30mg o-phenylenediame dihydrochloride (P8412, Sigma), and 200µl

was added to each well. The plate was wrapped in foil to protect from light and incubated for 30 min at room temperature. After this time, 50µl stop solution (concentrated sulphuric acid [$2NH_2SO_4$] diluted 1:10 in de-ionised water) was added to the wells and the contents briefly combined using a plate shaker. Spectrophotometry was subsequently performed using a plate reader set to 495nm as described for the commercial ELISA kits detailed in Section 2.6. The intra- and inter- CVs for this ELISA were 6.7% and 10%, respectively[5].

To calculate the amount of androstenedione and estradiol present in the follicular fluid, the concentration from the RIA and ELISA, respectively, was standardised to the follicular size as follows:

- Using the mathematical formula to calculate the volume of a sphere ($4/3 \times \pi r^3$) the volume inside the follicle was:

Follicle vol. (mm^3) = (4/3) x (3.14159265) x ((diameter of follicle/2) ^{3})

- Follicle volume (mm^3) was converted to volume (ml):

Follicle vol. (ml) = vol. (mm^3) x 0.001

- To calculate total amount (ng) of hormone in PBS and follicle fluid solution, the concentration measured was divided by the volume of the follicle (ml) plus 1ml PBS that the fluid wad diluted in:

Total amount (ng) = conc. (ng/ml; RIA/ELISA) x (1ml + follicle vol (ml))

- The total amount in the follicle (ng) was calculated by the total amount (ng) in solution divided by the follicle vol (ml):

Total amount in the follicle (ng) = total amount (ng)/follicle vol. (ml)

These calculations give the amount of either androstenedione or estradiol in each follicle expressed as ng/follicle.

[5] The estradiol ELISAs were performed by Sarah Henderson

For both culture media (androstenedione) and follicular fluid (androstenedione and estradiol) analyses, data were categorised into <3mm, 3-4mm and >4mm follicle diameter sizes. Values from individual follicles were used for comparison between control and treatment cohorts and for each treatment group the sample size was between n = 9 and 25 follicles for the <3mm group, n = 5 and 8 follicles for the 3-4mm group and n = 4 and 5 for the >4mm group.

7.2.4.3 Thecal cell mRNA extraction, cDNA synthesis and qRT-PCR

Thecal mRNA from ≥3mm follicles was extracted using the RNeasy Micro Kit (Qiagen Ltd.). Thecal cells stored in RNAlater (Applied Biosystems) were briefly spun, the RNAlater removed and 50µL RLT Buffer containing 1% β-mercaptoethanol was added to lyse the cells. One volume of 70% ethanol was added and the contents mixed and transferred to an RNeasy MiniElute spin column (Qiagen Ltd.). Subsequent purification steps were performed using the Qiagen RNeasy Micro kit. RNA was eluted with 14µL RNase free water (Qiagen Ltd.) and stored at -80°C[6]. RNA concentration was measured using a NanoDrop 1000 spectrophotometer (Fisher Scientific Ltd.) as described in section 2.8.1.4.

For the purpose of making cDNA from theca cells the SuperScript VILO cDNA synthesis kit (Invitrogen) was used instead of the standard kit described in other sections. This kit has been developed to give improved cDNA yield which is necessary when starting out with small amounts of RNA, and also to improve the efficiency of subsequent qRT-PCR so that low abundance target genes are amplified with greater sensitivity. In a 20µL reaction volume, 100ng RNA was added to the mixture. Reaction components and volumes are listed in Table 7.3 and cDNA was synthesised in a thermocycler (G-Storm GS1, GRI Ltd.) programmed for incubation at 25°C for 10 min, then 42°C for 1 h and finally 85°C for 5 min to terminate the reaction and stored at -20°C.

[6] RNA extraction from thecal cells was carried out by Dr. Julia Young.

Reagent	Volume (µL)	Final Conc.
VILO Reaction Mix	4	1X
SuperScript Enzyme Mix	2	1X
RNA	variable	100ng
NF H_20	variable	-
Final Volume 20µL		

Table 7.3 SuperScript VILO cDNA synthesis. The reagents supplied in the SuperScript VILO cDNA synthesis kit (Invitrogen) and volumes required for cDNA synthesis of thecal cells are detailed.

QRT-PCR was performed using SYBR Green reagents (Applied Biosystems) as described in Section 2.8.6.2 using 384-well reaction formats. A full list of genes examined in thecal cells and corresponding primer sequences are detailed in Table 7.4. Thecal cell gene expression was examined in all follicles collected >3mm and the subsequent analysis was carried out comparing expression in follicle sizes of 3-4mm and >4mm. Individual follicle gene expression values were utilised and for each treatment group the sample size ranged from n = 5 to 7 in the 3-4mm group and n = 4 to 5 in the >4mm group.

7.2.4.4 Statistical analyses

The semi-quantitative analysis of follicle number and all the data gathered from qRT-PCR gene expression experiments, where the differences between two means were assessed, were statistically analysed using a student's unpaired t-test assuming equal variances. In the instances where data were not normally distributed (unequal variances) a non-parametric Mann Whitney test was alternatively employed. For the thecal cell culture and follicular fluid hormonal assessment data were treated in the same way as above when comparing C and TP-treated groups or analysing the effect of changing follicle size. When analysing the effect of LH stimulation on thecal cell hormone output within a treatment group (before and after), a paired t-test was applied for repeated measures. These values are presented as mean ± sem. Data collected from the qualitative assessment of follicular proliferation and atresia by immunohistochemical staining, or the proportional assessment of non-estrogenic and

estrogenic follicles were subjected to a chi (χ^2) squared test. These values were presented as a percentage. P values of $P<0.05$ were considered statistically significant.

Gene (Accession No.)	Forward Sequence	Reverse Sequence	Product Size (bp)
AR (XM_001253942)	GCCCATCTTTCTGAATGTCC	CAAACACCATAAGCCCCATC	233
ERβ (NM_001009737.1)	GAGGCCTCCATGATGATGTC	GGTCTGGAGCAAAGATGAGC	208
LHR (NM_214449.1)	TCCGAAAGCTTCCAGATGTT	GAAATCAGCGTTGTCCCATT	199
IGF1R (XM_002696504.1)	CCAAAACCGAAGCTGAGAAG	TCCGGGTCTGTGATGTTGTA	199
INSR (XM_002688832.1)	CACCATCACTCAGGGGAAAC	CAGGAGGTCTCGGAAGTCAG	247
CYP11A (NM_001093789.1)	CAACGTCCCTCCAGAACTGT	CAGGAGGCAGTAGAGGATGC	172
HSD3B1 (NM_001135932)	GGAGACATTCTGGATGAGCAG	TCTATGGTGCTGGTGTGGA	200
StAR (NM_001009243)	GCATCCTCAAAGACCAGGAG	CTTGACACTGGGGTTCCACT	194
CYP17 (NM_001009483)	AGACATATTCCCTGCGCTGA	GCAGCTTTGAATCCTGCTCT	215
INHBA (NM_174363)	GAAGAGACCCGATGTCACCCAGC	TGTCGTCCTCTATCTCCACGTACCCG	113
INHBB (NM_176852)	ATGGCCTGGCCTCCTCCCG	CTTCAGGTAGAGCCACAGGCTGGC	101
INHA (NM_174094)	GAGCCCGAGGACCAAGATGTCTCC	CCTCAGCCTCTCCAGCATCTGGC	91
B-Glycan (NM_214272)	AATGGCACGCACTTCATTTT	GTCCCCTGGAAATCCGTTAT	186
Id1 (NM_001097568)	TCTGGGATCTGGAGTTGGAG	ATACGATCGTCCGCTGGAA	151
Id2 (NM_001034231)	CATCTTGGACTTGCAGATCG	AGAGAGCTTTGCTGTCATTTG	185
Id3 (NM_001014950)	ACTCAGCTTAGCCAGGTGGA	TTTGGTCGTTGGAGATCACA	160
GAPDH (NM_001034034)	GGCGTGAACCACGAGAAGTATAA	AAGCAGGGATGATGTTCTGG	229

Table 7.4 Thecal cell gene analysis using SYBR Green qRT-PCR. Gene names and accession numbers, forward and reverse primer sequences (5'-3') and PCR product sizes are listed. INSR: insulin receptor.

7.3 Results

7.3.1 The Lamb Ovary

7.3.1.1 Lamb ovaries are multifollicular and morphology is not a consequence of prenatal androgenisation

Lamb ovarian weight was not altered by prenatal androgenisation. Crude morphological examination of the 3-month lamb ovary, by assessment of H&E stained sections, revealed a multifollicular state with 10-30 large antral stage follicles routinely observed per section (Fig. 7.2). Control ovaries from both maternally and fetally exposed offspring exhibited this morphology and there was no clear relationship for antral follicle number between those ovaries of control and prenatally androgenised offspring. Representative whole ovary images from each treatment group are illustrated in Figure 7.2.

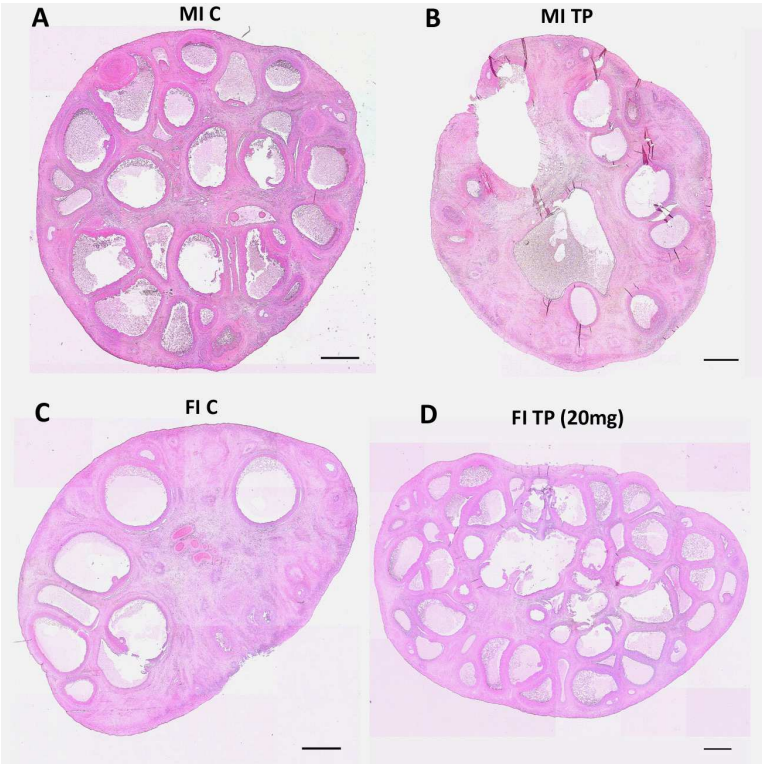

Figure 7.2 Lamb ovarian morphology. H&E staining did not reveal differences in the number of antral follicles between control and prenatally androgenised cohorts in the ~3 month lamb. Images represent maternal C (A) and TP (B) and fetal d62/72 C (C) and TP 20mg (D) ovarian sections. Scale bars are 100µm.

7.3.2 The Adult Ovary

7.3.2.1 Prenatal androgenisation has subtle effects on follicle number

Unlike the multifollicular phenotype observed in lamb ovaries in which large follicles were often situated throughout the medullary and cortical stroma, the young adult ovaries exhibited a settled appearance with approximately 2-8 large antral follicles situated in the peripheral ovarian cortex (Fig. 7.3). Gross inspection of adult ovary sections stained with H&E did not reveal stark differences between control and TP-exposed ovaries, nor was PCO-like morphology observed in any of the treatment groups assessed (representative images are illustrated in Fig. 7.3).

To investigate the follicular phenotype further, a detailed assessment was performed to assess follicle numbers over the stages of development by counting the number of primordial, primary, pre-antral and antral follicles (illustrated in Fig. 7.1) present in a representative portion of the ovary. A thorough stereological assessment of maternal control and TP exposed and fetal control and TP (4mg dose) treated animals was conducted. The number of primordial follicles present in the adult ovary was unaltered following both maternal and fetal TP exposure (Fig. 7.4 A and C, respectively). Similarly, maternal TP treatment did not influence primary, pre-antral or antral follicle number (Fig. 7.4.B). Direct fetal androgenisation did tend to increase in the number of antral follicles compared to control ovaries (Fig. 7.4.D), although this trend did not quite reach statistical significance (P=0.053). Primary and pre-antral follicle numbers were not affected by direct prenatal androgenisation (Fig. 7.4.D).

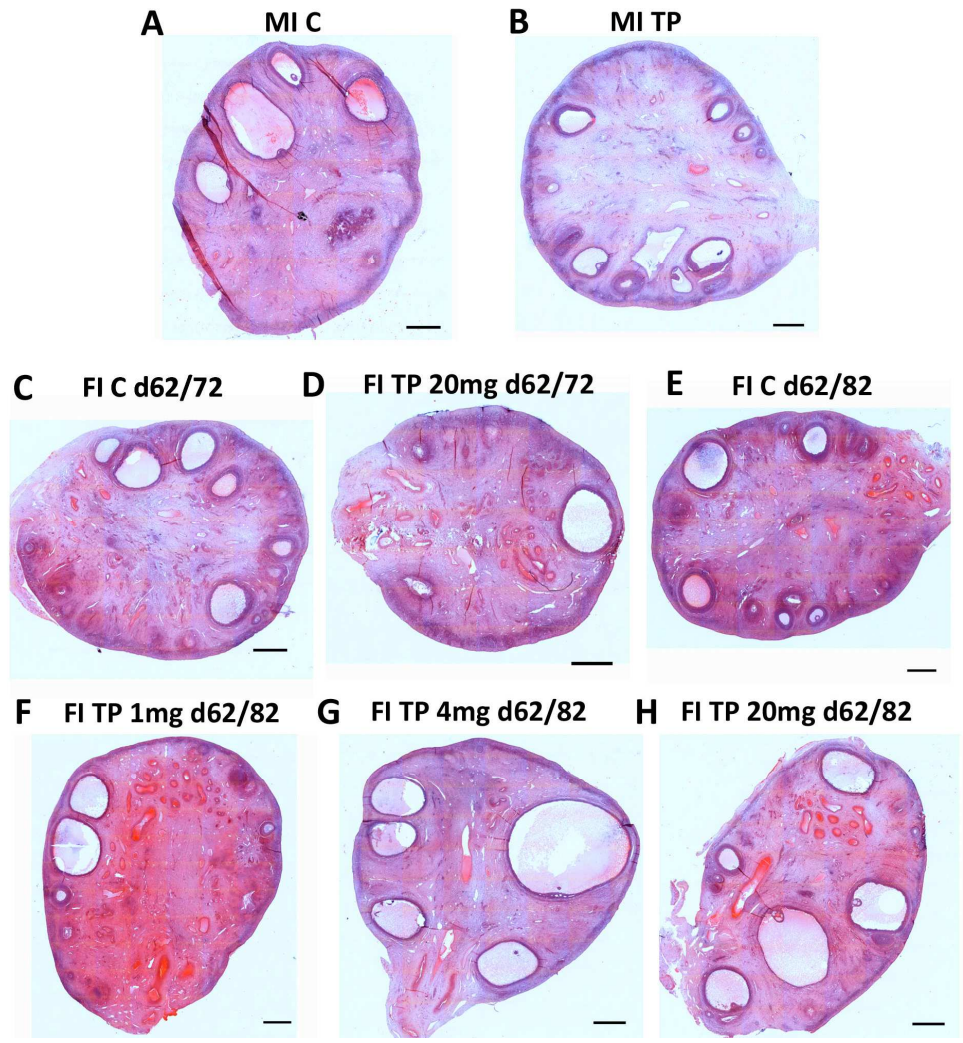

Figure 7.3 Adult ovarian morphology. Representative H&E images of ovarian sections from each treatment cohort. Images represent ovaries from maternal C (A) and TP (B), fetal d62/72 C (C) and TP 20mg (D), and fetal d62/82 C (E), TP 1mg (F), TP 4mg (G) and TP 20mg (H) *in utero* treated animals. Scale bars are 100µm.

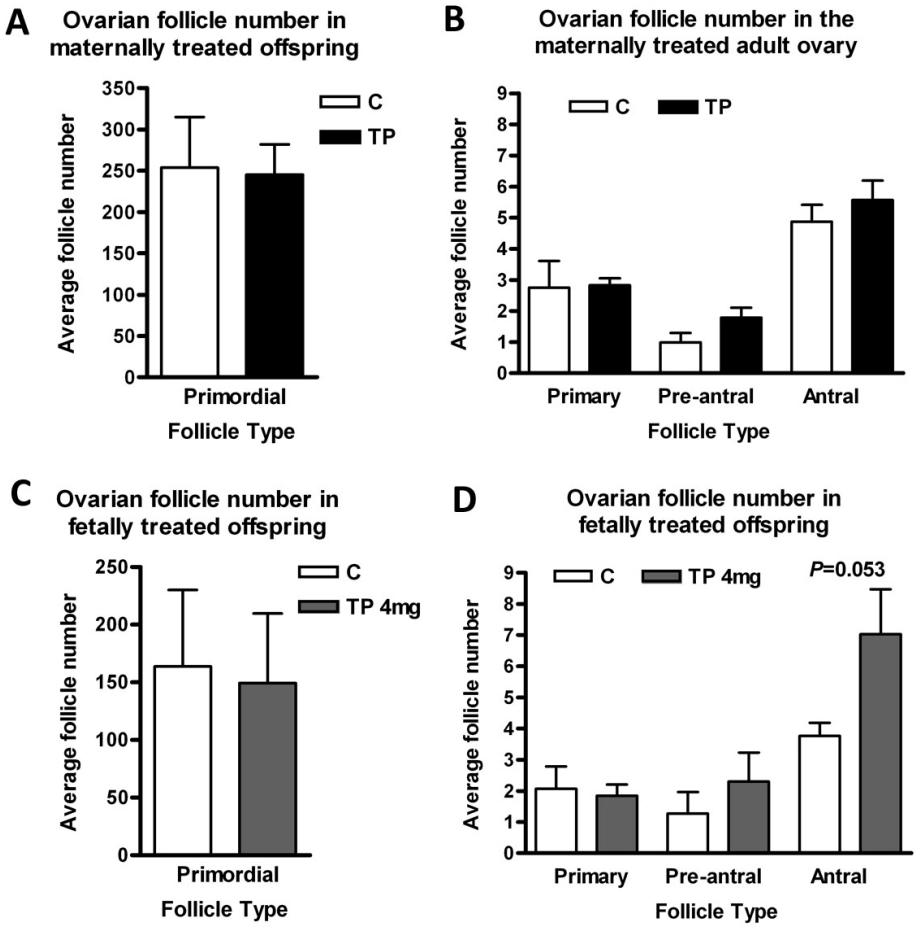

Figure 7.4 Follicle counts in the prenatally androgenised young adult sheep ovary. In maternal (A and B) and fetal d62/82 (C and D) treated cohorts the numbers of primordial, and primary, pre-antral and antral follicles were measured, respectively, by serial section analysis.

7.3.2.2 Follicle regression may be dysregulated in the prenatally androgenised ewe

Analysis of follicle proliferation and cell death was performed in the young adult ovary by immunohistochemical staining with markers for the activated cell cycle (Ki67) and apoptosis (cleaved caspase-3). A representative image of Ki67 immunostaining of an ovine antral follicle is shown in Figure 7.5.A and B. Figure 7.5.A depicts a non-growing follicle (grey arrow) adjacent to a growing follicle (black arrow). Positive nuclear staining is revealed throughout the granulosa and thecal cells of the growing follicle (Fig. 7.5.A and increased magnification in Fig. 7.5.B). In atretic follicles cleaved caspase-3 immunostaining was predominantly localised to the granulosa cell layer (Fig. 7.5.D and increased magnification in Fig. 7.5.E) with much less positive staining observed in the thecal layer.

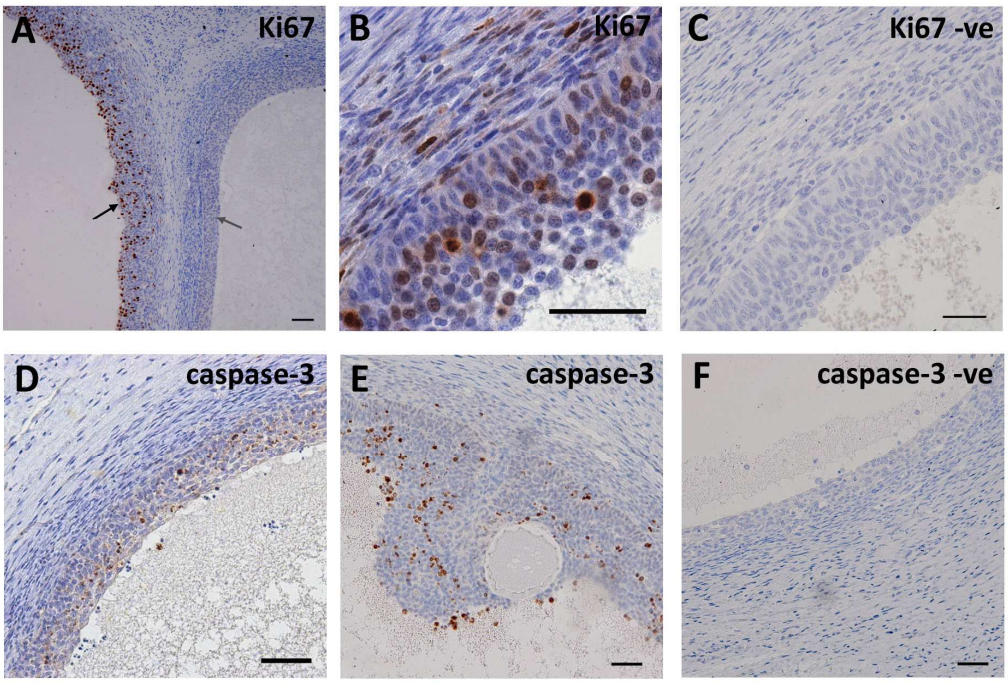

Figure 7.5 Immunohistochemical staining (brown) for Ki67 and cleaved caspase-3 in the adult ovary. Adult ovaries were stained for the proliferative marker, Ki67 (A and B), and the cell death marker, caspase-3 (D and E), to assess the proportion of follicle growth and regression, respectively. Negatives in C and F relate to Ki67 and caspase staining experiments, respectively. Scale bars are 100µm (A), 20µm (B and C) and 50µm (D-F).

In the same selection of follicles described in Section 7.3.2.1 (counted follicles) an in depth examination of follicle proliferation and atresia was carried out to assess the effect of maternal TP and fetal TP (4mg dose) exposure on follicle dynamics. In young adult ovaries, between 50-70% of antral follicles were growing and individual variation in this number was not related to indirect or direct prenatal androgenisation (Fig. 7.6.A and C, respectively). Of the total number of counted follicles the number of follicles with cell death suggesting atresia observed in control ovaries from maternal and fetal treatment regimes was 16% and 21%, respectively (Fig. 7.6 B and D). Prenatal androgenisation resulted in an increase in antral follicle atresia whereby the number of caspase positive antral follicles was increased following maternal (P<0.05; Fig. 7.6.B) and fetal (P<0.001; Fig. 7.6.D) TP exposure, doubling the respective figures to 32% and 38%.

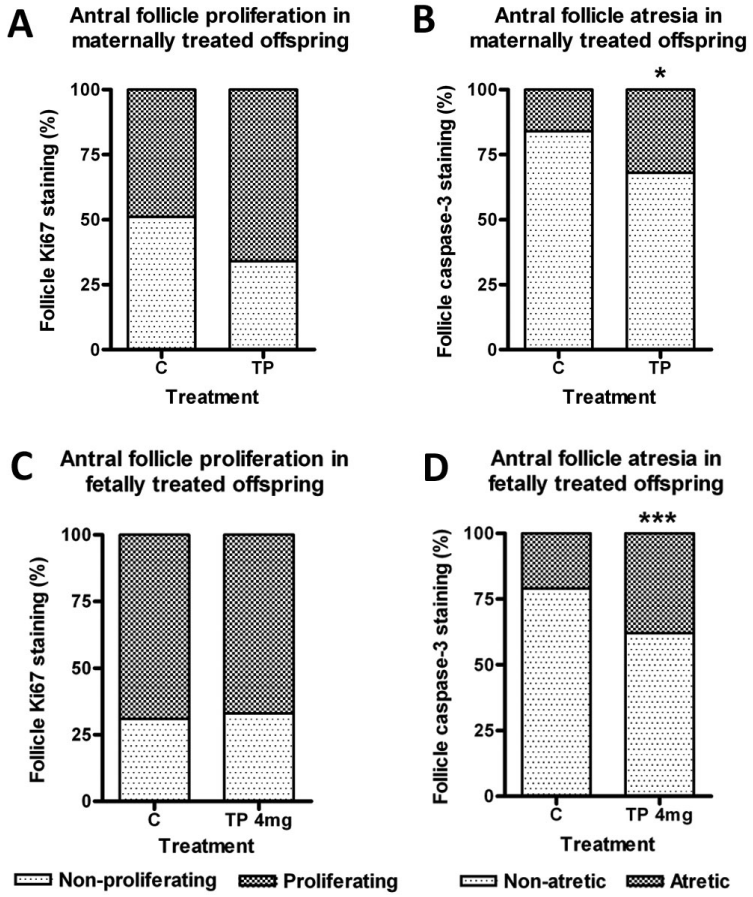

Figure 7.6 Antral follicle growth and atresia in the adult ovary of prenatally androgenised offspring. Growth (Ki67) and atresia (caspase-3) were determined by follicle immunostaining in maternal (A and B) and fetal d62/82 (TP 4mg; C and D) androgen exposed offspring, respectively. * P<0.05, *** P<0.001.

Analysis of the estrogenic status of the follicle was another approach utilised to assess the level of follicular degeneration occurring in the ovary. These experiments were carried out in the maternal TP and fetal TP 20mg treated cohorts, in which the follicular fluid from individual follicles was collected and the concentration of estradiol measured. A cut-off value of 30 ng/follicle estradiol was applied to distinguish between non-estrogenic (healthy) and estrogenic (not growing or dying) follicles. In both small (<3mm; $P<0.05$) and large (>3mm; $P<0.05$) follicles, there were significantly more estrogenic follicles in the maternal TP-exposed animals compared to controls (Fig. 7.7.A). The presence of more healthy follicles suggests that even though there was increased atresia in these ovaries, overall, there was no negative impact on the healthy growing follicles. There was no significant treatment effect of fetal TP exposure on the proportion of non-estrogenic to estrogenic follicles (Fig. 7.7.B).

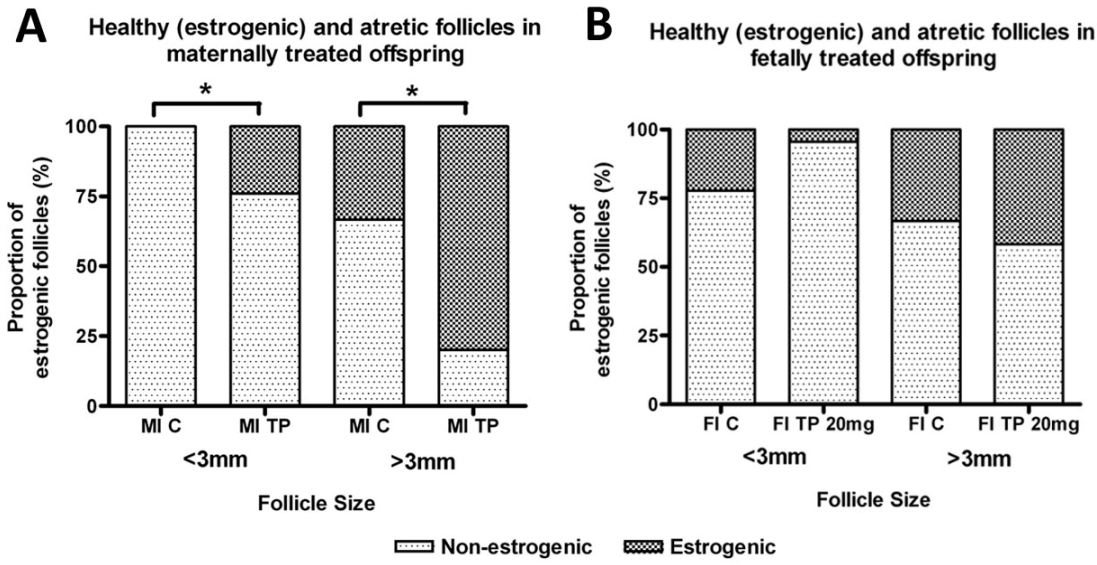

Figure 7.7 The proportion of non-estrogenic and estrogenic follicles in the prenatally androgenised ewe ovary. Estradiol was measured in the follicular fluid of antral follicles by ELISA and estrogenic and non-estrogenic follicles were determined by a cut-off value of 30 ng/follicle. Analysis was carried out in maternal TP (A) and fetal TP 20mg (B) treated offspring. * $P<0.05$.

7.3.2.3 Thecal androstenedione output is increased by prenatal androgenisation *in vivo* and *in vitro* and as a consequence of fetal injection

The amount of androstenedione secreted by follicles was assessed by measurement of this hormone directly in the follicular fluid collected from each follicle. Over increasing follicle size the amount of androstenedione present in the follicle fluid was significantly reduced in control (maternally treated) animals (<3mm Vs 3-4mm, $P<0.01$ and 3-4mm Vs 4mm, $P<0.05$; Fig. 7.8.A). The negative relationship in the amount of androstenedione present in the follicular fluid and follicular size is thought to reflect conversion of this steroid into estradiol as the follicles mature. This inverse relationship was also found in the fetally treated control cohort (Fig. 7.8.B), however, androstenedione secretion compared to the maternally treated control cohort was in fact increased by approximately 3-fold in <3mm ($P<0.05$), 3-4mm ($P<0.01$) and >4mm ($P<0.01$) follicles (Fig. 7.8.B).

Assessment of the effect of prenatal androgenisation in the young adult revealed that maternal TP-exposure led to an increase in androstenedione levels in the follicular fluid of the largest follicles (>4mm) although this did not quite reach statistical significance ($P=0.055$; Fig. 7.8.C). A similar non-significant trend was found in the fetally TP-exposed cohort in >4mm follicles ($P=0.15$; Fig. 7.8.D) in which there was a great deal of individual variation. Nevertheless the difference in the amount of androstenedione produced in fetally TP treated >4mm follicles was 35-fold greater compared to the maternal control cohort.

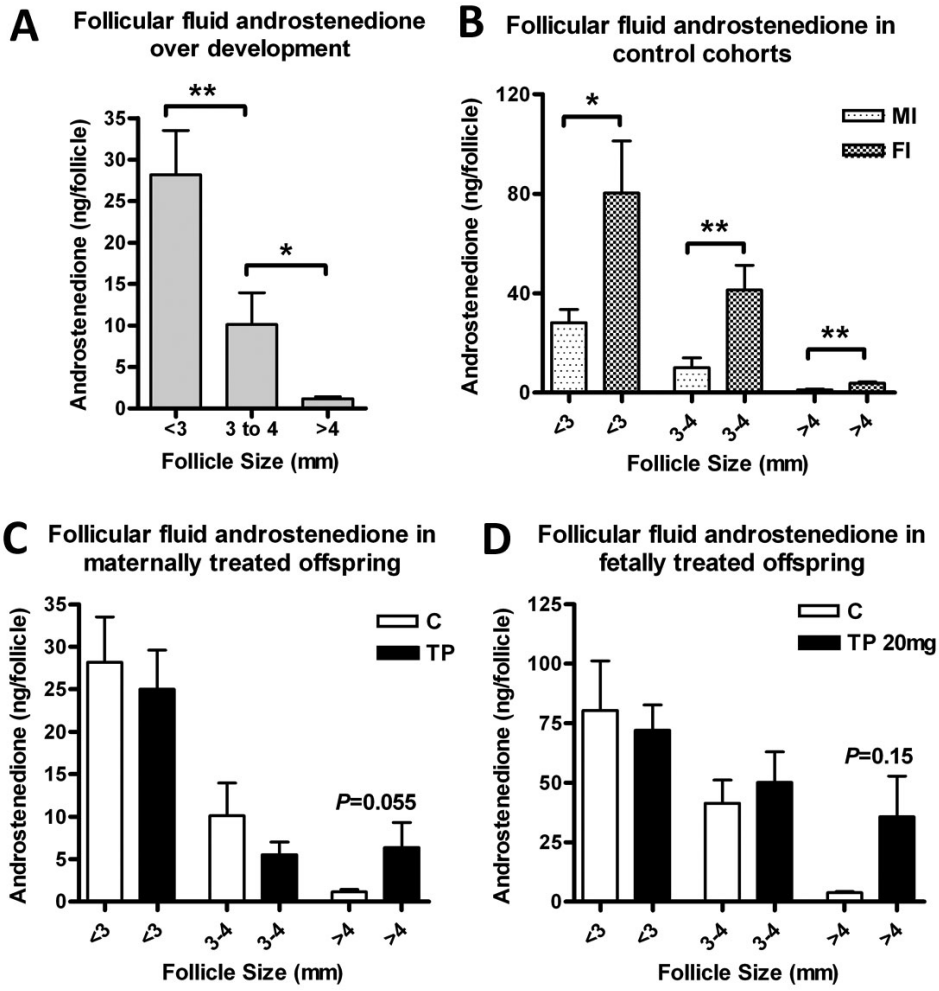

Figure 7.8 Androstenedione measurement in the follicular fluid of prenatally androgenised ewes. Androstenedione was measured by RIA in antral follicles that were <3, 3-4 and >4 mm in diameter. The concentration of androstenedione over increasing follicle size was compared in control animals (A), and between control treatment cohorts (B). The effect of prenatal androgenisation was assessed in maternally (C) and fetally d62/82 (TP 20mg; D) exposed offspring. * $P<0.05$ and ** $P<0.01$.

In order to gain insight into the mechanism behind the effect of prenatal TP on thecal androstenedione secretion, the estrogenic status of the follicle was additionally taken into account. In estrogenic follicles androstenedione secretion, *in vivo*, would be expected to fall due to conversion of this steroid into estradiol. In fact, while there was no difference between androstenedione concentration in >4mm follicles when non-estrogenic and estrogenic groups were compared in control cohorts, androstenedione concentration did show a trend to be reduced in estrogenic, compared to non-estrogenic follicles, of maternally and fetally TP-exposed groups (Fig.7.9.A and C, respectively). As shown in Figure 7.8.C and in Figure 7.9.A, the amount of androstenedione in follicular fluid of >4mm follicles was increased in maternal TP-exposed animals compared to controls. There was also a trend for increased androstenedione concentration in >4mm follicles from fetally TP treated offspring (Fig. 7.8.D and Fig. 7.9.C). When follicle androstenedione concentrations from maternally and fetally treated ewes were split into groups for follicles that were and were not producing estradiol, androstenedione appeared to be most increased in the non-estrogenic follicles (Fig. 7.9A and C, respectively).

Therefore, it was hypothesised that in these follicles there is less conversion of androstenedione to estradiol. This did appear to be the case in the maternally TP-treated ewes (Fig. 7.9.B), in which there was a trend for reduced estradiol concentrations in the follicular fluid of >4mm follicles. Conversely, in the fetally TP treated cohort, the opposite trend was in place and there was increased estradiol concentrations in >4mm non-estrogenic follicles from the TP cohort (Fig. 7.9.D). The segregation of follicles in to these groups resulted in a small sample size so these observations are trends and not entirely conclusive, however, this provides some evidence that in the maternal treated cohort there could be a defect in the conversion of androgens to estrogens in these follicles, which is not present in the fetally injected cohort. Unfortunately due to the lack of available good quality granulosa cell mRNA it was not possible to measure the gene expression of *CYP19* to determine whether, even in the presence of increased substrate, that it is the catalytic conversion of steroids that is defective in the maternally treated animals.

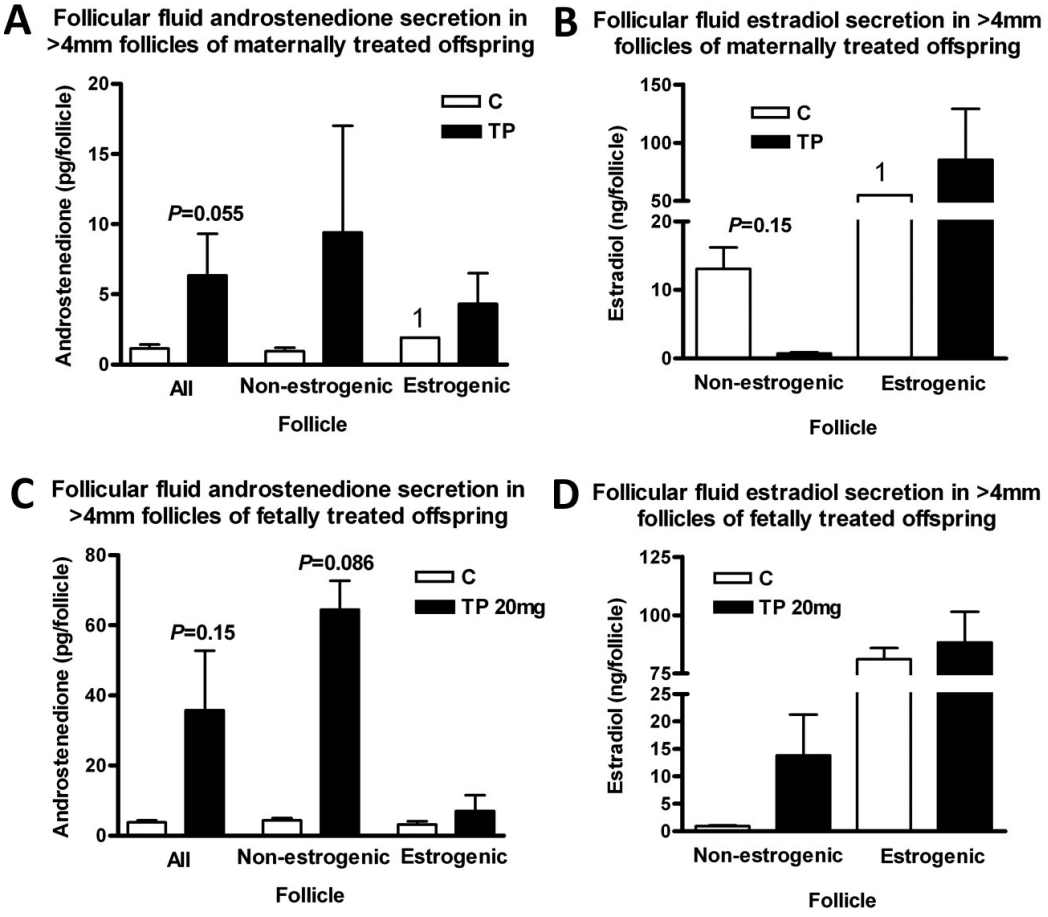

Figure 7.9 Androstenedione and estradiol concentrations in follicle fluid of non-estrogenic and estrogenic follicles. The concentration of androstenedione and estradiol was compared between non-estrogenic and estrogenic follicles in maternal (A and B, respectively) and fetal d62/82 (C and D, respectively) C and TP cohorts. 1 = 1 animal in group.

The thecal cell capacity to produce androstenedione following prenatal androgenisation was also determined *in vitro*. Androstenedione production and secretion was measured after follicle culture pre- and post-LH stimulation. Under both culture conditions (-/+ LH) the concentration of androstenedione in culture media increased with follicle size in maternally treated cohorts, although the difference was more pronounced in TP treated animals (-LH, <3mm Vs 3-4mm; $P<0.001$ and 3-4mm Vs >4mm; $P<0.05$, +LH, <3mm Vs 3-4mm; $P<0.001$ and 3-4mm Vs >4mm; $P<0.05$) than control animals (non-significant; Fig. 7.10.A). In the maternal treated cohort the thecal cells responded to the addition of LH with either a trend or significant increase in androstenedione output in both control and TP-treated groups (indicated by asterisks in Fig. 7.10.A). Maternal prenatal androgenisation resulted in an

enhanced response to LH in >4mm follicles compared to control LH stimulated >4mm follicles (P<0.05). In fact, androstenedione secretion for LH stimulated control >4mm follicles was similar to the unstimulated TP >4mm follicles (Fig. 7.10.A). In the fetally treated cohort, androstenedione output was significantly increased between <3mm and >4mm sized follicles in controls pre-LH stimulation (P<0.01), but this trend was less apparent in TP-treated animals, before stimulation, and in control and TP-treated animals post-LH stimulation (Fig. 7.9.B). LH stimulation only marginally increased androstenedione secretion across the treatment groups and the concentration was in fact reduced post LH in control 3-4mm and >4mm follicles (Fig. 7.9.B), although these alterations were not significant.

Maternal prenatal androgenisation was found to have a bimodal effect on androstenedione secretion depending on the size of the follicle both before and after LH stimulation (Fig. 7.10.C). In the smallest follicles (<3mm) prenatal TP-exposure led to a suppression in thecal androstenedione output basally (P<0.05) and after stimulation with LH (P<0.05). Conversely, androstenedione secretion was increased non-significantly in 3-4mm follicles and significantly in >4mm follicles from TP-exposed animals, during culture, before (P=0.095) and after (P<0.05) the addition of LH (Fig. 7.10.C). In the fetally androgenised cohort there was no association between prenatal androgens and androstenedione secretion in small or large follicles (Fig. 7.10.D). In fact, like the findings in the follicular fluid from these animals, the capacity to produce androstenedione was increased in thecal cells from fetally treated compared to maternally treated animals (Fig. 7.10.E). Although this trend was present post-LH stimulation this effect was most pronounced pre-LH stimulation in <3mm (P<0.05), 3-4mm (P<0.01) and >4mm (P<0.01) follicles (Fig. 7.10.E). In the fetal treated cohort the lack of response to LH and no effect of prenatal androgenisation could therefore be a result of a system already producing androstenedione at full capacity.

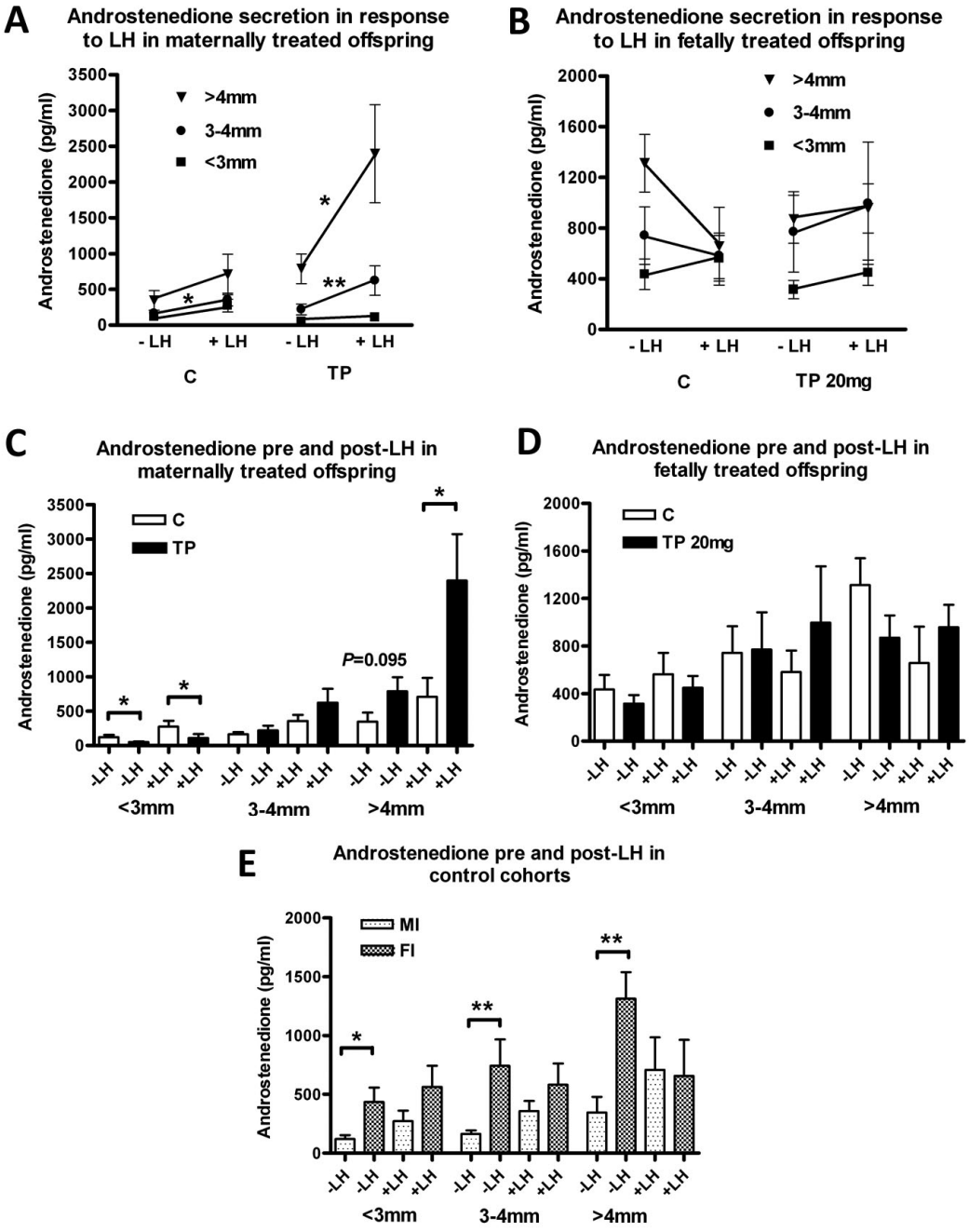

Figure 7.10 Androstenedione measurement in culture media from thecal cells of prenatally androgenised ewes. Androstenedione concentration was measured in media pre- and post-thecal cell stimulation with LH. The response to LH is depicted as line graphs for control and treated groups in maternally (A) and fetally (B) androgenised ewes. The effect of prenatal androgenisation was compared in maternally (C) and fetally d62/82 (TP 20mg; D) treated animals. The effect of treatment administration route was determined in control cohorts (E). Asterisks (*) above the joining line in (A) represent a significant difference between pre- and post-LH stimulation within the treatment group. * $P<0.05$ and ** $P<0.01$.

7.3.2.4 Prenatal androgenisation programmes increased thecal cell steroidogenic capacity and receptivity to LH

Thecal cell gene expression analysis for a range of genes involved in the steroidogenic pathway was carried out in individual follicles, to determine whether intrinsic changes in thecal function could contribute to enhanced androstenedione synthesis. Follicles were grouped into size categories of 3-4mm and >4mm, to determine if expression changes were present earlier, or occurred later, in follicle development, or if they were present at all large follicle sizes (≥3mm). Analysis revealed that maternal prenatal androgenisation led to a global up-regulation of steroidogenic genes in ovine thecal cells from young adults. Maternal TP-exposure resulted in an up-regulation in thecal *StAR* mRNA from ≥3mm follicles ($P<0.01$), with the largest increase observed in >4mm follicles ($P=0.051$; Fig. 7.11.A). There was also a trend for increased thecal *CYP11A* across the follicle groups (Fig. 7.11.D). Similarly, *HSD3B1* ($P<0.01$) and *CYP17* ($P<0.05$) transcripts were also up-regulated in theca from ≥3mm follicles (Fig. 7.11.G and J, respectively).

Direct fetal treatment led to changes in steroidogenic gene expression that were related to both prenatal androgens and also to the delivery method itself. Fetal TP-exposure resulted in an up-regulation of thecal *StAR* in 3-4mm follicles ($P<0.05$), however this was not altered in >4mm follicles (Fig. 7.11.B). In fact, *StAR* was significantly increased in fetally treated control compared to maternally treated control >4mm follicles ($P<0.05$; Fig. 7.11.C), indicating that both control and TP thecal cells are over-expressing *StAR*. In the fetally treated cohort, there was a trend for elevated thecal cell *CYP11A* expression in ≥3mm follicles of TP-exposed animals (Fig. 7.11.E). In this instance the fetal control injection did not differ to the maternal control animals (Fig. 7.11.F). Thecal *HSD3B1* was non-significantly altered by fetal androgenisation (Fig. 7.11.H), although the pattern of expression was similar to thecal *StAR*. There was an increase in 3-4mm follicle thecal *HSD3B1* expression following fetal TP-exposure (Fig. 7.11.H) and an increase in *HSD3B1* mRNA in both control and TP-exposed cohorts in >4mm follicles compared to maternal controls (Fig. 7.11.H and I). This pattern was also repeated for *CYP17* gene expression in which fetal TP-exposure significantly up-regulated *CYP17* in 3-4mm follicles ($P<0.05$) and both control and TP treatments resulted in increased *CYP17* in >4mm follicles (Fig. 7.11.K). The fetal injection itself resulted in a significant increase in *CYP17* expression in >4mm follicles when compared to maternal treated controls ($P<0.05$; Fig. 7.11.L).

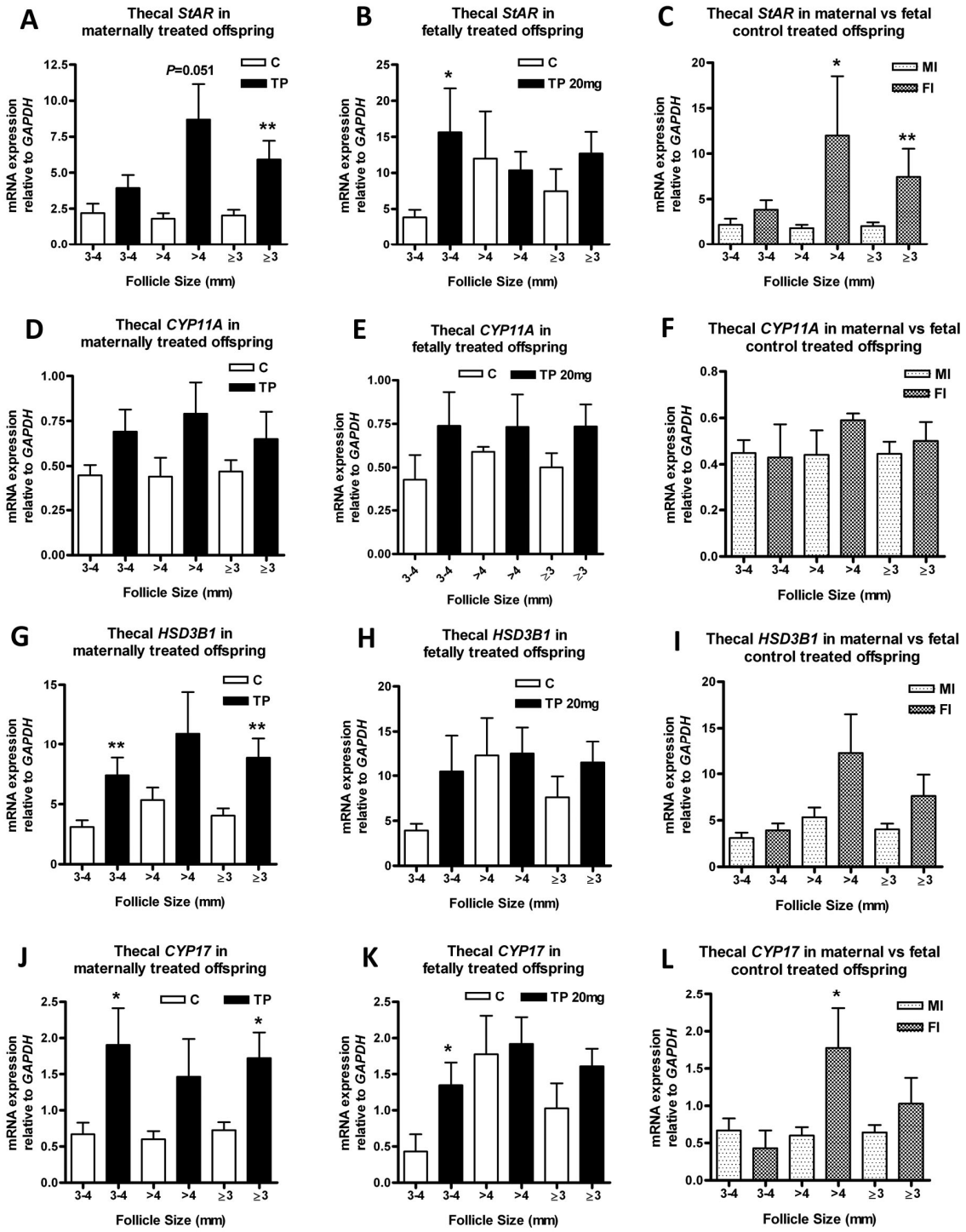

Figure 7.11 The steroidogenic gene expression in thecal cells of prenatally androgenised ewes. The effect of prenatal androgenisation on thecal cell steroidogenic capacity was determined by qRT-PCR. In maternally and fetally d62/82 treated animals and in control cohorts respectively, the genes assessed were *StAR* (A-C), *CYP11A* (D-F), *HSD3B1* (G-I) and *CYP17* (J-L). * $P<0.05$ and ** $P<0.01$.

The ability of thecal cells to respond to hormonal signals was examined, by qRT-PCR analysis of a number of hormonal receptors, in follicles from prenatally androgenised sheep. In all follicle sizes assessed (≥3mm) neither maternal nor fetal prenatal TP-exposure led to alterations in the thecal mRNA expression of *AR* (Fig. 7.12.A and B, respectively), *ERβ* (Fig. 7.12.C and D, respectively) or *INSR* (Fig. 7.12.E and F, respectively). Maternal prenatal androgenisation did however lead to a significant increase in the expression of thecal *LHR* in ≥3mm follicles ($P<0.05$), that was present in both 3-4mm follicles ($P<0.05$) and in >4mm (non-significant) follicles (Fig. 7.12.G). A trend for increased *LHR* in 3-4mm follicles was also observed in fetally TP-exposed animals (Fig. 7.12.H), and in this instance there was no additional effect exerted by the fetal injection itself (Fig. 7.12.I).

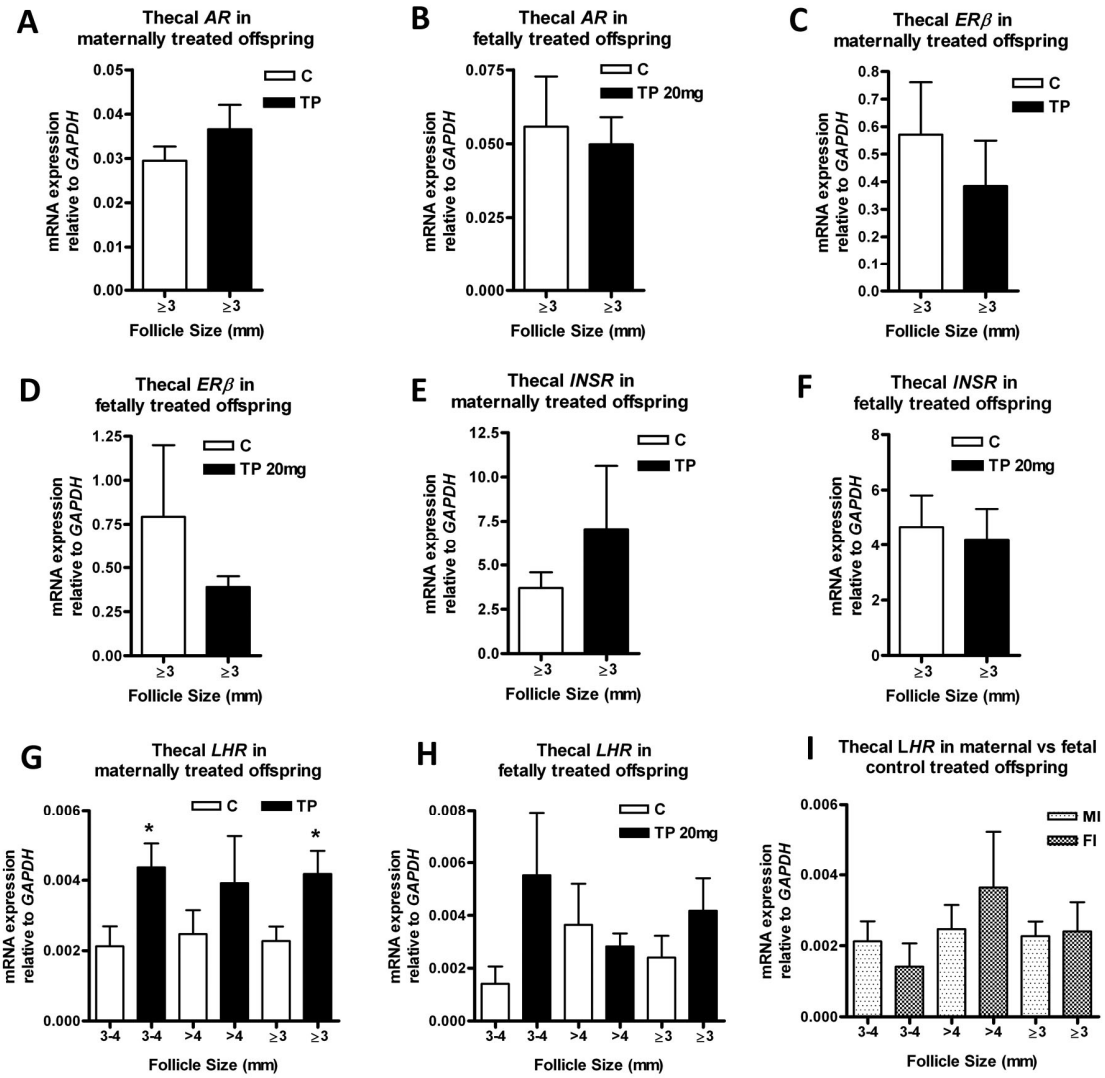

Figure 7.12 The receptor gene expression in thecal cells of prenatally androgenised ewes. The effect of prenatal androgenisation on thecal cell hormonal receptivity was determined by qRT-PCR. In maternally and fetally d62/82 treated animals respectively, the genes assessed were *AR* (A and B), *ERβ* (C and D), *INSR* (E and F), and *LHR* (G and H). The effect of the fetal injection on *LHR* expression is shown in I). * $P<0.05$.

7.3.2.5 Thecal cell expression of genes involved in growth and hormone secretion are affected by prenatal androgenisation

Other genes that can affect follicle growth as well as the availability of steroid hormones were analysed including the activins and inhibins. The three subunits that make up these dimeric proteins, including *INHA*, *INHBA* and *INHBB* were analysed by qRT-PCR. There was a non-significant trend for increased thecal *INHA* and *INHBA* subunit mRNA expression, and *INHBB* was up-regulated ($P<0.05$), in 3-4mm follicles in maternally TP-exposed animals (Fig. 7.13.A, D and G, respectively). Conversely, fetal TP-exposure had no effect on *INHBB* mRNA expression in thecal cells (Fig. 7.13.H), nor was there an effect of fetal injection treatment (Fig. 7.13.I). However, the fetal control injection did lead to a non-significant downward trend in both thecal *INHA* and *INHBA* subunit expression in 3-4mm follicles when compared to maternal controls (Fig. 7.13.C and F, respectively). Fetal prenatal TP-exposure appeared to rescue this decrease in thecal *INHA* and *INHBA* in 3-4mm follicles (Fig. 7.13.B and E, respectively), although large individual variation existed.

Thecal cell expression of the inhibin receptor β-Glycan (TGFβR3) was also examined. No changes were observed in the expression of thecal *β-Glycan* mRNA in either maternally (Fig. 7.14.A) or fetally (Fig. 7.14.A) TP-exposed animals at any follicle size analysed, nor was there an apparent effect of fetal treatment delivery (Fig. 7.14.C). IGF1 signalling was additionally assessed in the thecal cells of prenatally androgenised sheep. Thecal *IGF1R* mRNA expression was increased in ≥3mm follicles in maternally TP treated animals ($P<0.05$), this effect being particularly prominent in 3-4mm follicles ($P<0.05$; Fig. 7.14.D). Fetal prenatal androgenisation did not affect thecal *IGF1R* expression (Fig. 7.14.E), however in this case the fetal injection itself resulted in a significant up-regulation of *IGF1R* transcript ($P<0.05$, >4mm follicles; Fig. 7.14.F) indicating an increase in *IGF1R* in both fetally control and TP-treated animals (Fig. 7.14.E).

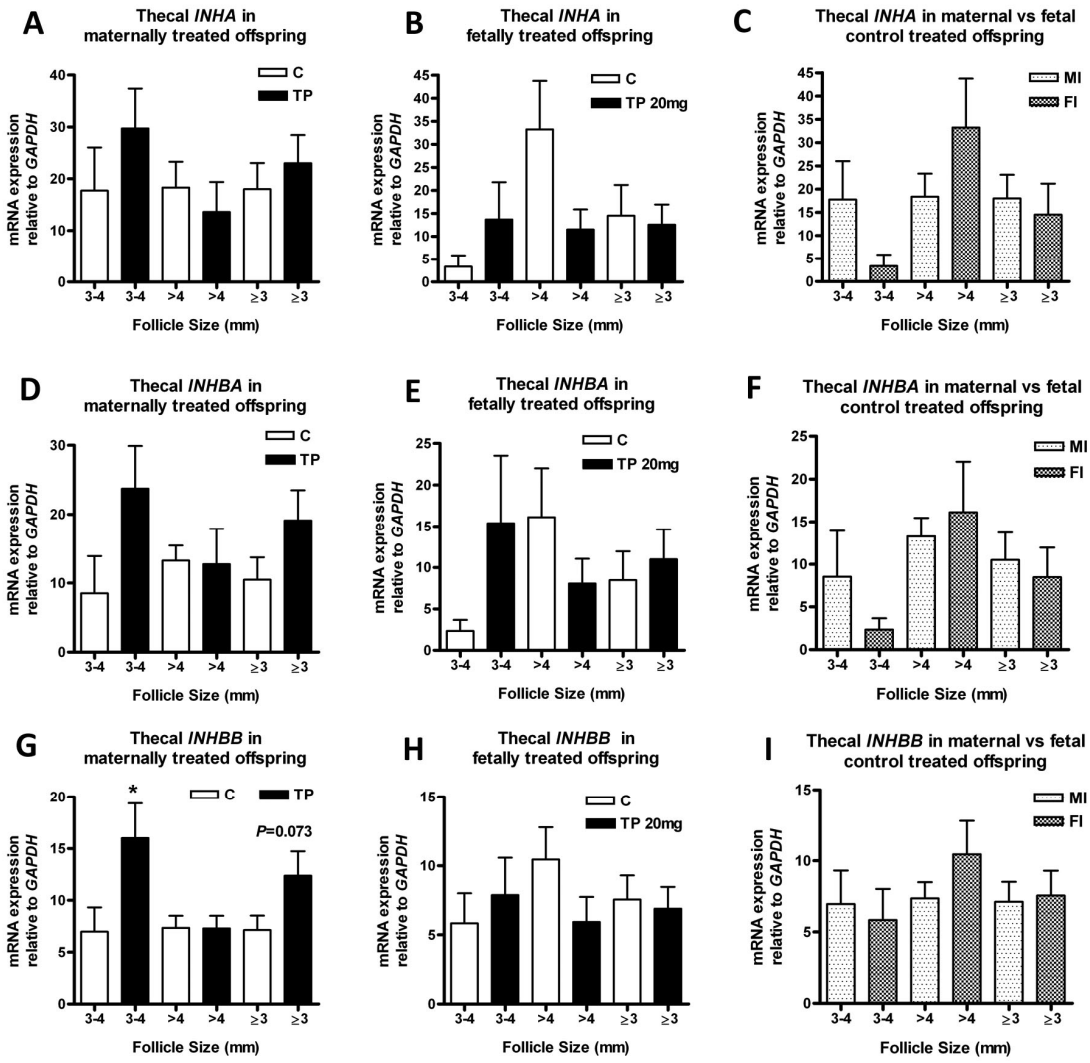

Figure 7.13 The activin and inhibin subunit gene expression in thecal cells of prenatally androgenised ewes. The effect of prenatal androgenisation on thecal cell inhibin subunit expression was determined by qRT-PCR. In maternally and fetally d62/82 treated animals and in control cohorts respectively, the genes assessed were *INHA* (A-C), *INHBA* (D-F) and *INHBB* (G-I). * $P<0.05$.

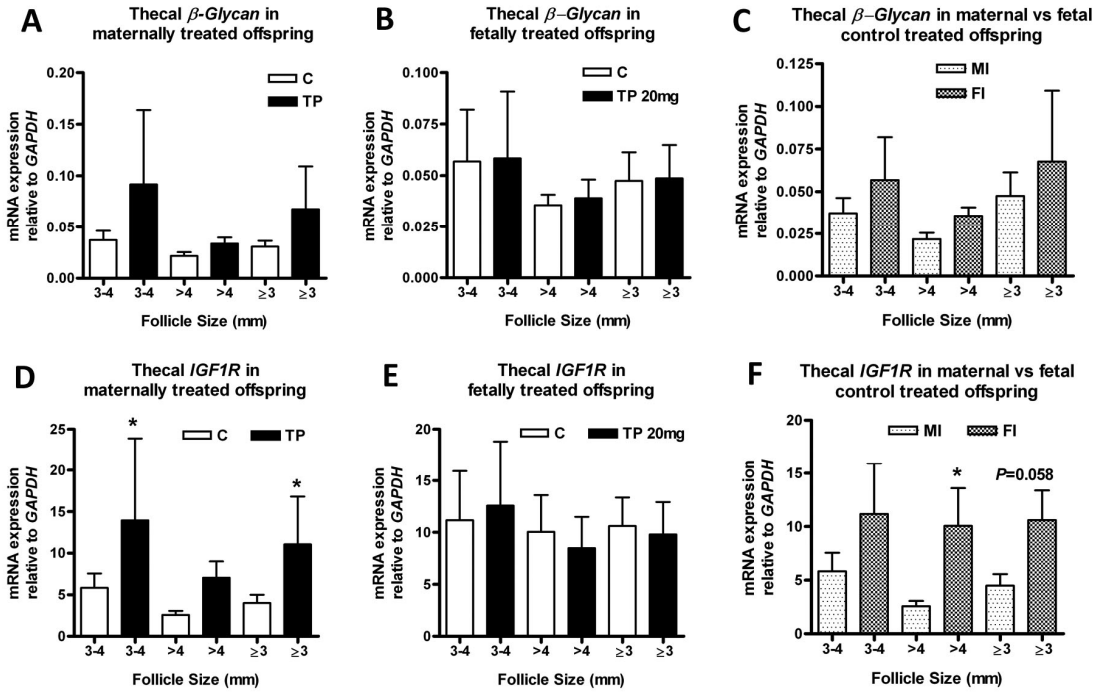

Figure 7.14 *β -Glycan* and *IGF1R* gene expression in thecal cells of prenatally androgenised ewes. The effect of prenatal androgenisation on thecal cell gene expression was determined by qRT-PCR. In maternally and fetally d62/82 treated animals and in control cohorts respectively, *β-Glycan* (A-C) and *IGF1R* (D-F) gene expression was assessed. * $P<0.05$.

7.3.2.6 The expression of the Id1 in thecal cells is differentially altered in the fetal treatment cohort

The Id genes (*Id1-3*) were analysed in the thecal cells of prenatally androgenised offspring. *Id1* was more abundantly expressed than *Id2* (Fig. 7.15) and *Id3* was not detected at the mRNA level in these cells. The expression of thecal *Id1* was unaltered by maternal androgenisation (Fig. 7.15.A) and fetal androgenisation led to a down-regulation of this gene in >4mm follicles ($P<0.05$; Fig. 7.15.B). However, the fetal control cohort were found to have a significantly higher levels of *Id1* than the maternal control group (Fig. 7.15.C), so that the down-regulation by fetal TP exposure resulted in comparable *Id1* expression to the maternal treated animals. There was no alteration in the expression of *Id2* in any of the groups analysed (Fig. 7.15.D-E).

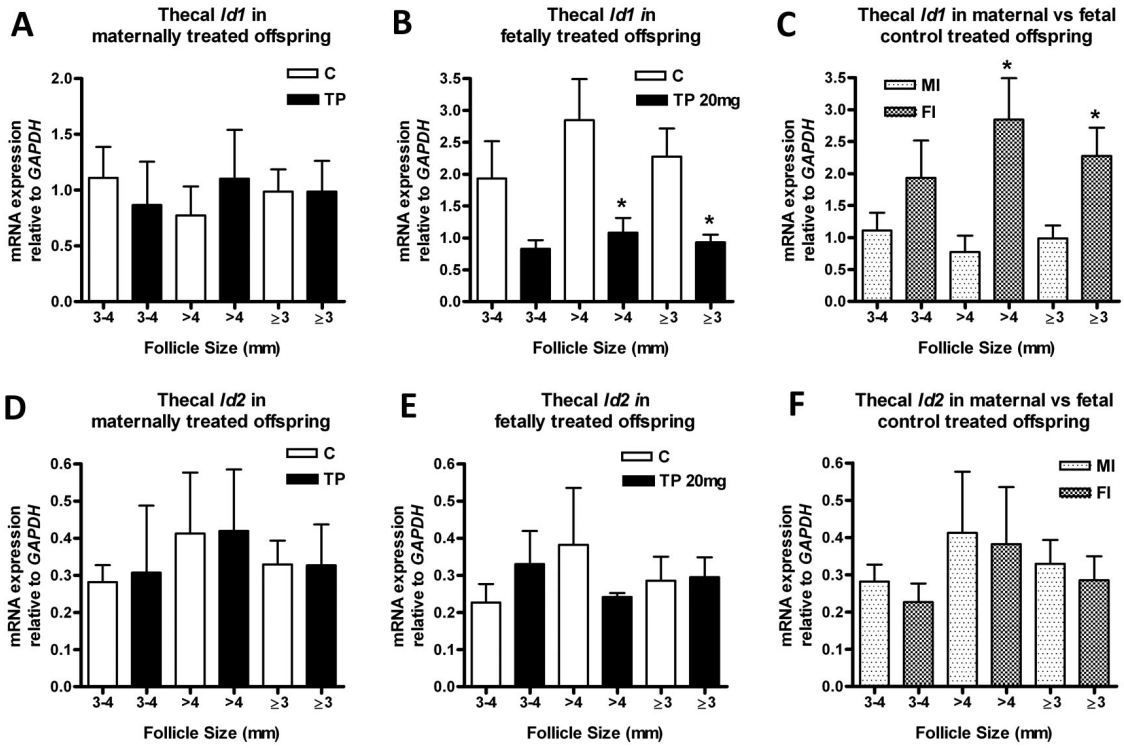

Figure 7.15 Id gene expression in thecal cells of prenatally androgenised ewes. The effect of prenatal androgenisation on thecal cell gene expression was determined by qRT-PCR. In maternally and fetally d62/82 treated animals and in control cohorts respectively, *Id1* (A-C) and *Id2* (D-F) gene expression was assessed. * $P<0.05$.

7.4 Discussion

In the sheep prenatal androgenisation model, West *et al.* reported that polyfollicular ovaries were present in 5 week old lambs that were exposed to testosterone, but not DHT, from d30-90 of pregnancy (301). The present study found that while some ~3 month old lamb ovaries were highly polyfollicular, containing densely packed antral follicles, this was independent of treatment group and in fact there was a great deal of individual variation concerning the number of follicles present. Indeed, there is a surge of follicular growth in the neonatal lamb at 2-4 weeks in which there is a peak of growing follicles followed by a wave of atresia (599, 600), which would explain this multifollicular phenotype in these young lambs. In young adults this generalised polyfollicular morphology was lost and ovaries were found to contain fewer large antral follicles.

While crude assessment did not appear to identify distinct differences in ovarian morphology as a result of prenatal androgenisation, other studies have suggested that folliculogenesis is disrupted in both women with PCOS (317, 318) and in the prenatally androgenised sheep model (332, 333) through alterations in follicular recruitment and regression. Therefore an in depth stereological examination of ovarian serial sections was performed to elucidate whether subtle differences occurred in response to prenatal androgens in terms of follicle number and also the level of follicle growth and atresia. Neither maternal nor fetal prenatal androgenisation resulted in changes in primordial cell number in ovaries from 11 month animals. Additionally no differences were found in the number of primary, pre-antral or antral follicles across the treatment groups although there was a non-significant trend for an increased antral follicle number in those animals fetally exposed to 4mg TP. Furthermore, in line with the unaltered follicle number the proportion of growing follicles, as assessed by follicles positive for the cell cycle marker, Ki67, was unaffected in both treatment cohorts. A study by Steckler *et al.* investigated the effect of prenatal androgenisation from d30-90 on ovarian reserve in the d140 fetal ovary, and reported that there was decreased primordial follicle number (332), which was speculated to be a result of increased follicular recruitment of primordial follicles into transitional and primary follicles. The same affect is also shown in the d30-90 prenatally androgenised 8 month old animal, however not in the 20 month old ewe (333). These studies are in agreement with human studies investigating follicular dynamics in the PCOS ovary, in which an increase in primary and antral follicle number has been reported, parallel to a decrease in primordial follicle number (317).

It is likely that these differences in follicular recruitment and growth between this study and previous studies are associated with the different treatment periods of *in utero* androgenisation. Indeed if these alterations were to occur through the androgenic actions of TP then the increased trend for antral follicle number present in the fetally d62-102 treated cohort, in which fetal testosterone levels are greatly increased compared to the maternal TP cohort, may reflect the longer period of treatment in previous studies (d30-90). In fact, DHT but not TP exposure from d30-90, was found to increase the number of small follicles in adult sheep ovaries (335). In terms of regulating follicle number, this might suggest that the duration of treatment or amount of androgen that the fetus is exposed to is more significant than the timing of treatment. Certainly germ cell number is not affected by maternal prenatal androgenisation in the d90 fetal ovary following exposure from d60-90 of gestation (Chapter 4), whereas changes in primordial follicle number are reported by d140 as a result of a longer 60 day exposure to androgens (332).

Further to this, studies in sheep have found a reduction in follicular atresia that is associated with the estrogenic programming effects of prenatal androgenisation. Steckler *et al.* demonstrated that during culture of ovarian biopsies from d30-90 prenatally androgenised sheep, those animals exposed to TP, but not DHT, exhibited a reduction in follicle regression, although there was no difference between control and treatment cohorts prior to culture (335). The present study also found alterations in the amount of follicular atresia measured by the counting of cleaved caspase-3 positive follicles in ovarian serial sections. The fact that these changes were observed in maternal and fetal treated animals also suggests that there might be a direct androgenic programming element to the regulation of follicle degeneration. This is in contrast to observations of Steckler *et al.* and other studies using human PCOS ovaries where there was a reduction in follicular atresia (319). Given the observation that not all of these ewes were cycling during this time, implying that some of these animals were still immature, and the ovaries were not yet polycystic in morphology, the changes in the rate of atresia may reflect intrinsic alterations in folliculogenesis that is occurring in the prenatally androgenised cohorts, that is not yet affected by extra-ovarian factors such as the gonadotrophins.

In addition to measuring antral follicle atresia by caspase-3 immunostaining as an indicator of cell death in fetal TP 4mg exposed animals, the estrogenic status of follicles was also assessed in the fetal TP 20mg cohort. In contrast to the finding of increased atresia in

maternally treated animals, the estrogenic status of follicles from these animals suggested that there was an increased number of healthy follicles, particularly in those follicles that were >3mm. This would in fact indicate more follicular activity and reduced follicular regression in the ovaries of these animals and this fits with previous literature that also proposes this mechanism in PCOS-models (319, 334, 335). Of course these fetal TP cohorts are slightly different in that a lower and higher dose of TP administration was assessed between experiments. This was due to limitations in the time available to assess all of the animals. Nevertheless, the data presented could suggest that at least in the maternal treated cohort, while more follicles are estrogenic and appear healthy, more follicles are also programmed for cell death, resulting in a scenario whereby a possible phenotype for increased follicle number is cancelled out by increased follicle atresia in the young adult. Speculatively, intrinsic ovarian mechanisms may balance out the altered follicular dynamics to prevent a multifollicular phenotype from arising. As these animals were assessed at a young age it is possible that these mechanisms would be absent in a more phenotypically mature animal, thus leading to the increase in follicle number reported elsewhere (334, 335).

It may be that the increase in follicle recruitment and growing follicle number, opposed to the decrease in follicle regression, reported in some human and sheep studies, may differentially occur through respective androgenic and estrogenic actions. Indeed, the short term treatment of cycling adult Rhesus monkeys with testosterone or DHT, result in increased preantral and small antral follicle number and follicular growth, although follicular atresia was not affected by either treatment (336). It is clear that the high level of heterogeneity characteristic of PCOS could account for some of the differences encountered between studies. For example, Maciel *et al.* reported an increased number of primary through to antral follicles in PCOS ovaries however did not find any alterations in primordial follicle reserve or in the level of follicular atresia in PCOS (318).

As well as evaluating the intrinsic ovarian features that could alter morphology, the steroidogenic potential of the ovary was also examined to determine whether changes associated with PCOS were present. Systemically there was no alteration in circulating sex steroids, including estradiol, testosterone or androstenedione as a result of prenatal androgenisation (Chapter 6). Hyperandrogenaemia is however a cardinal feature of PCOS of which the ovary is a contributor. The thecal cells are responsible for androgen biosynthesis and synthesise androstenedione when triggered to do so by LH, which is subsequently

converted to estradiol by granulosa cells in the presence of FSH. In the prenatally androgenised ewe, this study aimed to assess the local production of androstenedione by the thecal cell *in vivo* and *in vitro* and to examine whether the steroidogenic capacity is altered by changes in thecal cell gene expression.

Androstenedione was measured *in vivo* to give a physiological assessment of thecal cell steroidogenic output. In the largest antral follicles (>4mm) androstenedione concentration, measured in the follicular fluid, was increased in both maternally and fetally treated cohorts as a result of prenatal androgenisation, although in both cases this followed a non-significant trend. Crucially the impact of the fetal injection was apparent: in fetal control follicles there was a trend for increased androstenedione output compared to maternal control follicles at all sizes assessed and this was significant for follicles >4mm. Therefore the androstenedione concentration was in fact increased by 35-fold in these large follicles from fetal TP-exposed animals when compared with maternal controls. Similarly, the increased capacity for thecal cell androstenedione production in prenatally androgenised animals was demonstrated *in vitro* by measurement of androstenedione in media from thecal cell cultures pre- and post-LH stimulation. Maternal treatment resulted in an increased drive for androstenedione production in large (>4mm) follicles, basally and post LH stimulation. Interestingly in small (<3mm) follicles androstenedione secretion was decreased both basally and post LH in TP-exposed animals compared to controls, the opposite of the effect in >4mm follicles. These data are in accordance with increased androstenedione production from cultured thecal cells from women with PCOS, before and after LH stimulation (344, 345), and may indicate changes in ovarian steroid production that are not yet identifiable systemically. In the fetally treated cohort there was, like the *in vivo* assessment, a universal enhancement of androstenedione concentration in media collected from cultured thecal cells at all follicle sizes assessed. The thecal cell response to LH was more limited than the maternal treatment groups and there was no added effect of prenatal androgenisation.

While there was some evidence in the maternally androgenised cohort that increased androstenedione levels are a consequence of reduced conversion to estradiol, these studies have demonstrated a clear thecal cell phenotype that would promote increased steroidogenic drive. Previous research that has assessed the factors that might lead to increased steroid production in human PCOS thecal cells have identified alterations in gene expression related to the steroidogenic biosynthesis pathway. These include the up-regulation of *StAR* (598),

CYP11A and *CYP17* (345, 598) as well as increased enzymatic activity of 17α-hydroxylase, 3β-HSD and 17β-HSD (345). Assessment of the prenatally androgenised ovine model via the maternal route of TP administration identified the up-regulation of many genes involved in steroid biosynthesis including *StAR*, *CYP11A* (trend), *HSD3B1* and *CYP17*. Furthermore thecal *LHR* gene expression was increased in maternal TP-exposed animals, a finding that has also been reported in human thecal cells from PCOS patients (598). These data imply that the enhanced androstenedione secretion could also be mediated through both increased sensitivity to LH and increased activity of the steroidogenic pathway.

In the maternal TP exposed young adult ewe, thecal expression of the inhibin subunit *INHBB* was significantly increased in 3-4mm follicles compared with controls. This is in contrast to reports by West *et al.* 2001 who found a non-significant downward trend for *INHBB* mRNA expression by *in situ* hybridisation in follicles from d30-90 prenatally androgenised 5-week lambs. However, aside from the differences between these studies in terms of prenatal treatment period and the age of animals assessed, *INHBB* expression in the study by West *et al.* included the granulosa cells which are the predominant producer of activin and inhibin. In this study the increase in the *INHBB* subunit in thecal cells could lead to an increase in activin AB or activin B (not inhibin B which is not produced in sheep). Also the increase, albeit non-significant, in *INHA* and *INHBA* subunit expression could potentially lead to increased activin A or inhibin A protein expression. The fact that activin is known to suppress androstenedione expression, while inhibin enhances it (120-122), makes these findings difficult to reconcile. Nevertheless it is possible to speculate that the increase in inhibin A could contribute to the enhanced androstenedione synthesis that occurs in the prenatally androgenised sheep, although inhibin subunit changes were found in the 3-4mm follicle and androstenedione was not raised until the >4mm size.

The role of the *Id* genes as novel candidates for the regulation of thecal cell function was also assessed in the ovine model of prenatal androgenisation. The Id genes have roles in proliferation, apoptosis and differentiation and are regulated by members of the transforming TGFβ superfamily, including the BMPs and activin. Although the *Id* genes are generally described in either development or in cancer, we have shown that they are also present in the adult ovary and can be regulated by TGFβ members (Chapter 5). Due to the role in inhibition of differentiation and promotion of proliferation, the Id genes are potential candidates for the dysregulation of folliculogenesis observed in PCOS. In fact, we have shown that in the ovine

prenatal androgenisation model there are alterations in Id1 and Id3 protein expression in the fetal ovary. Id1 and Id3 were up-regulated in the d70 and d90 fetal ovary following maternal exposure to androgens and Id3 was up-regulated at d70 in the fetally exposed group (Chapter 4). In the prenatally androgenised adult the thecal expression of *Id2* was unaltered. *Id1* was down-regulated as a result of fetal TP-exposure; however, the control group in this cohort presented a phenotype in which *Id1* was elevated in the first instance. Since activin can regulate the Ids, changes in Id expression may have been reflected in the local activin production however no changes were observed in the expression of the inhibin subunits *INHBA* or *INHBB*. Furthermore, the finding that Id protein expression is down-regulated in the thecal layer of atretic follicles in the adult ovary (Chapter 5) might predict a down-regulation of the *Id* genes in the thecal cells of non-estrogenic follicles; however this correlation was not observed. Therefore the effect of the *Id1* gene expression change in the fetally exposed cohort is not easily reconciled, but further indicates a global dysregulation of gene expression in the thecal cells of this cohort.

This chapter has highlighted a number of interesting pathways that are influenced by prenatal androgenisation and may provide insight into the mechanisms involved in PCOS. There was evidence for subtle alterations in follicular dynamics as a result of increased *in utero* androgens that were present in an otherwise normal ovarian morphology. Interestingly, there was variation in the amount of ovarian activity, observed by the number of antral follicles present in ovaries from different individuals; however, these morphologies were not linked to treatment. Two explanations for the lack of an ovarian morphological phenotype may be the shorter period of prenatal androgenisation compared to other studies in the sheep and also the assessment of these parameters in the young sheep in the first breeding season opposed to more mature animals in their second breeding season. Much of the changes observed in sheep studies have occurred in the second breeding season. This provided a unique model in which it was possible to dissect out intrinsic ovarian changes that were present in the absence of an altered hormonal environment and, at this stage, disrupted folliculogenesis.

Indeed, it was demonstrated that there are intrinsic ovarian abnormalities that are programmed by prenatal exposure to androgens, which are not associated with an altered steroid or gonadotrophin hormone environment. This study has for the first time characterised thecal cell androgen production and steroidogenic gene expression in the ovine

prenatal androgenisation model and revealed a considerable increase in the amount of androgens produced by thecal cells which is likely driven by a universal up-regulation of genes involved in steroid biosynthesis. Moreover, an up-regulation of LHR in these same cells is an additional pathway in which androgen synthesis may be augmented.

Finally, injecting the fetus *in utero* can also lead to ovarian irregularities, paralleling some of the findings in the maternally TP exposed cohort. These included the increased expression of steroidogenic genes and also a vast enhancement of thecal cell androgen output far exceeding that of the maternal TP phenotype. While the mechanisms resulting in this are not clear it implies that the reproductive anomalies apparent in the maternally treated cohorts may not entirely be programmed by the effects of androgens and/or estrogen. Also the finding that *Id1* is increased in the thecal control cohort might provide insight into an alternative mechanism for the programming of follicle dysregulation that is not influenced by exposure to sex steroids.

In conclusion, mid-gestation prenatal androgenisation does not result in PCO, perturbed ovulatory cycles, or an altered hormonal environment in young adult ewes. Despite this, there are phenotypic differences present in the adult ovary that are likely programmed in fetal life to result in a pre-PCOS phenotype, observed at this stage. Therefore the thecal cell phenotype that is similar to that in women with PCOS may be inherent and not secondary to systemic environmental changes that are typical of PCOS. This is particularly remarkable given that thecal cells are not present at the time of prenatal treatment, so androgens must act on other cell types such as a thecal stem cell, somatic pre-granulosa cell or germ cell. Alternatively the developing thecal cell may be influenced by other environmental factors in the adult. Women with PCOS may also have increased adrenal gland androgen secretion. Therefore prenatal androgen exposure may have a similar effect in the adult adrenal gland as the ovary, to pre-dispose enhanced androgen production. In the following chapter, the effects on prenatal androgenisation on the adrenal are investigated.

Chapter 8

The effect of prenatal androgens on adrenal gland function

8.1 Introduction

In addition to the ovary, the adrenal gland is a major source of steroids including androgens and glucocorticoids, which are released upon stimulation by ACTH secreted by the pituitary gland. In females, approximately 50% of androgens are synthesised by the adrenal which produces, in order of abundance, DHEA, DHEAS, androstenedione and testosterone (393). This is of importance for women with PCOS, in which hyperandrogenaemia is thought to be a result of the increased secretion of androgens from both the ovary and the adrenal gland (408, 409, 411, 413, 414). Evidence for the increased adrenal expression of steroidogenic enzymes in these women suggests intrinsic changes to the adrenal to drive steroid production. In fact daughters of women with PCOS, that were therefore exposed to a androgenic maternal environment, were assessed peri-pubertally and had increased serum androgens and 17α-OH progesterone post-ACTH stimulation that would suggest increased activity of the enzyme 17α-hydroxylase (412). Accordingly, studies in the prenatally androgenised Rhesus monkey have demonstrated increased basal levels of DHEA (297, 415) and ACTH-stimulated production of DHEA and androstenedione (415), although in this instance there was no reciprocal increase in the level of 17α-OH progesterone (415). Given the increase in the ovarian capability to produce androgens in this ovine model, androgen output in the stimulated adult adrenal gland was also assessed.

The adrenal gland is also responsible for regulating the body's response to stressful stimuli, whether that is physical or emotional (386, 387, 390). In this way, the stimulation of glucocorticoids from the adrenal gland is crucial for maintaining physiological homeostasis and they can act directly on a variety of tissues including liver, muscle, fat, bone, the brain and the kidneys (31). It is therefore imperative that this system is controlled and triggered in an appropriate manner to maintain homeostatic equilibrium and normal body function. Adult diseases in which this does not happen, such as Cushing's syndrome in which there is over-production of cortisol, lead to obesity, altered fat distribution, muscle weakness and other metabolic irregularities that arise as result of the crucial role that cortisol has in maintaining these systems (31). In other syndromes, including PCOS, it is suggested that there is an enhanced response of the HPA axis to stressful stimuli and increased cortisol output (426) which, given some of the metabolic disturbances common to PCOS, may identify a pathway that contributes to this phenotype. These observations have also been extended to an animal model of PCOS, where prenatally androgenised non-human primates have been shown to

have increased ACTH-stimulated corticosterone production (but not cortisol) compared to control animals (415). The effect of prenatal androgens on HPA function in the adult sheep is not published but there are some indications that cortisol production is not affected in this model (427).

In the ovine fetus it is the maturation of the HPA axis that is crucial to the initiation of parturition and maturation of organs including the lungs, which is essential for survival (601). The adrenal glands are visible in the ovine fetus by gestational d25 and by d60 two morphologically distinct cortical zones are present (602). The zona glomerulosa is present at d80 and by d90 the inner cortical zone begins to resemble that of the zona fasciculata that becomes fully formed by d120 (602). The HPA axis matures at approximately d125 in the sheep fetus and with that there is a steady increase in cortisol levels due to increased adrenal steroidogenesis. This activation of fetal adrenal hormone production is stimulated by the pituitary hormone ACTH (603).

The ovine fetal adrenal secretes large amounts of cortisol between d40-90 and d120 to term, but during d90-120 cortisol production is low (604). In fact any cortisol present in the fetal circulation between d90-120 is thought to mainly come from the placental transfer from mother to fetus (605). The expression of adrenal steroidogenic genes reflects this triphasic pattern of cortisol production. Both *CYP11A* and *CYP17* are strongly expressed in the fetal adrenal between d40-60 and d120 to term but are down-regulated in between these times (398). Conversely, *CYP21* mRNA expression steadily increases throughout gestation, suggesting that the production of cortisol is also limited by enzymatic steps at the beginning of the pathway (398). In fact infusion of ACTH into the fetus at ~d100 stimulates cortisol secretion through the up-regulation of *CYP11A* and *CYP17*, and this treatment does not affect the expression of *CYP21* (603). Therefore ACTH directly controls adrenal steroid production by mediating specific adrenal gene expression and it is the availability of this pituitary hormone that controls the pattern of glucocorticoid production in the fetus.

The aim of this chapter is to assess the function of the adult adrenal gland in relation to steroid hormone output and adrenal gene expression in the ovine prenatal androgenisation model. The lamb adrenal is also assessed to determine the timing of manifestation of a phenotype. In addition to studying the post-natal effects of prenatal androgens, the fetal

adrenal gland was also assessed to determine whether early alterations during adrenal development might be reflected in the adult animal or whether fetal adrenal development was normal and any affects in the adult develop post-natally. In the lamb and adult prenatally androgenised offspring, the steroidogenic potential of the adrenal gland was examined by measuring: A) the ACTH-stimulated production of cortisol and adrenal androgens including androstenedione and testosterone, and B) the adrenal steroidogenic gene expression. In the fetus, the objectives were to: A) assess the effect of maternal androgenisation for either 10 or 30 days (commencing at d60), in the d70 and d90 fetal adrenal respectively, by measuring the expression of steroidogenic genes, and B) compare this with the effect of directly injecting the fetus once with 20mg TP at d60 and assessing the adrenal at d70.

8.2 Materials and Methods

8.2.1 Experimental animals

The female animals assessed in this chapter are detailed in Table 8.1.

Study	Treatment	Sample number (n)
Fetal		
Fetal Treatment	Injection at d60, assessed at d70	C = 4, TP 20mg = 8
Maternal Treatment	Treatment from d60-70, assessed at d70	C = 3, TP = 6
	Treatment from d60-90. assessed at d90	C = 6, TP = 8
Lamb		
Maternal Treatment	Treatment from d62-102	C = 9, TP = 7
Adult		
Fetal Treatment	Injection at d62 and 72	C = 4, TP 20mg = 8
	Injection at d62 and 82	C = 4, TP 1mg = 5, 4mg = 5, 20mg = 7
Maternal Treatment	Treatment from d62-102	C = 5, TP = 9

Table 8.1 The experimental animals included in the assessment of the adrenal gland, treatment regime and corresponding sample numbers.

8.2.2 Quantitative real time -PCR

The expression of a range of genes was investigated in the fetal, lamb and adult sheep adrenal gland. The methodology used for these experiments are as described in Section 2.8 of the Materials and Methods chapter, which details RNA extraction, cDNA synthesis, primer validation and SYBR Green qRT-PCR protocols that were utilised for assessment of this tissue. The primer details for each gene assessed are listed in Table 8.2.

Gene (Accession No.)	Forward Sequence	Reverse Sequence	Product Size (bp)
CYP11A (NM_001093789)	CAACGTCCCTCCAGAACTGT	CAGGAGGCAGTAGAGGATGC	172
HSD3B1 (NM_001135932)	GGAGACATTCTGGATGAGCAG	TCTATGGTGCTGGTGTGGA	200
StAR (NM_001009243)	GCATCCTCAAAGACCAGGAG	CTTGACACTGGGGTTCCACT	194
CYP17 (NM_001009483)	AGACATATTCCCTGCGCTGA	GCAGCTTTGAATCCTGCTCT	215
AR (XM_001253942)	GCCCATCTTTCTGAATGTCC	CAAACACCATAAGCCCCATC	233
ERα (NM_001001443)	GAATCTGCCAAGGAGACTCG	CCTGACAGCTCTTCCTCCTG	187
CYP11B (NM_174638)	AGAAGTACACGCCCTTGGTG	AGCGCGTGGATAAAGTTCAG	217
CYP21 (NM_174639)	CACATGTCTGTGGTGGACCT	AAGTGACTCGGGAGCATGAG	163
POMC (NM_001009266)	GAGAGCAGCCAGTGTCAGG	GAAATGGCCCATGACGTACT	159
GAPDH (NM_001034034)	GGCGTGAACCACGAGAAGTATAA	AAGCAGGGATGATGTTCTGG	229

Table 8.2 Adrenal gland gene analysis using SYBR Green qRT-PCR. Gene names and accession numbers, forward and reverse primer sequences (5'-3') and PCR product sizes are listed.

8.2.3 Determination of adrenal function

To assess adrenal function a synacthen test was performed at approximately 10 weeks and 10.5 months of age. Synacthen is a synthetic chemical equivalent to the endogenous pituitary secreted ACTH and is routinely used to investigate adrenal insufficiency. Animals were fasted on the day of the test, which was carried during late morning and early afternoon in groups of 8-10 animals at a time. The animals within treatment cohorts were randomly mixed in these test groups to avoid variability due to diurnal rhythms. A basal blood sample was drawn from the jugular vein prior to administration of a 2ml intravascular dose of synacthen (50µg/ml in saline; Alliance Pharmaceuticals Ltd., Wiltshire, UK) and subsequent sampling occurred 15 and 30 min post-stimulation. Blood was decanted into heparin-containing test-tubes, spun at 3000 rpm at 4°C for 15 min, and the supernatant stored at -20°C.

Hormonal analysis was carried out by RIA pre- and post-ACTH stimulation, and included assays for androstenedione, testosterone and cortisol. For the testosterone and androstenedione measurements, serum was extracted prior to the assay as described in Section 2.5.1. The general RIA protocol is detailed in Section 2.5.2 and specific information for each hormone assay is described in Appendix A. The reagent information and CVs are listed in Table 8.3.

Hormone	Primary antibody	Tracer (^{125}I labelled)	Standards	Intra and Inter Assay CVs (%)
Andro-stenedione	Rabbit anti-Androstenedione (07-109216; MP Biomedicals, Illkirch, France)	Andro-stenedione (07-109226)	Andro-stenedione (A9630)	4, 7.5
Testosterone	Rabbit anti- Testosterone (R3S07-259; AMS Biotechnology, Oxfordshire, UK)	Testosterone (07-189126)	Testosterone (T1268)	4, 8
Cortisol	Rabbit anti-Cortisol (20-CR5; Fitzgerald, MA, USA)	Cortisol (07-121126)	Corticosterone (C2505)	6, 9

Table 8.3 The hormones measured in plasma by RIA during a synacthen test. Listed are the primary antibodies, tracers and standards employed for each RIA. Radiolabelled tracer was supplied by MP Biochemicals, Illkirch, France and the standards by Sigma-Aldrich.

8.2.4 Statistical analyses

Where the differences between two means were assessed, including the gene expression and hormonal analysis following the synacthen test at each time point (0, 15 and 30 min), data were statistically analysed using a student's unpaired t-test assuming equal variances. If the data were not normally distributed (unequal variance) a non-parametric Mann Whitney test was alternatively employed. For the d62/82 experimental cohort in which there were numerous treatment groups, statisitcal analyses were conducted so that each TP-treated group (shaded bar) was compared to the control group (white bar). The values are presented as the mean ± sem. P values of $P<0.05$ were considered statistically significant.

8.3 Results

8.3.1 Adult adrenal function in the prenatally androgenised sheep model

In order to determine whether the adrenal gland of prenatally androgenised animals might function differently upon stimulation, ewes were delivered a large dose of a synthetic form of the pituitary hormone, ACTH, and the subsequent production of hormones monitored 15 and 30 min later. Cortisol, androstenedione and testosterone were assayed to determine how steroidogenic pathways might be affected by prenatal androgenisation. Those animals that were exposed to androgens via the maternal route did not show any differences in cortisol (Fig. 8.1.A) or androstenedione (Fig. 8.1.B) response to ACTH, however, they did have an exaggerated testosterone response to adrenal stimulation after 30 min ($P<0.05$; Fig. 8.1.C). Fetal androgenisation had no effect on adrenal steroidogenic output following stimulation with ACTH. Like the maternal treated cohort, the fetal treatment paradigm had no effect on the adrenal response to ACTH, for either glucocorticoid or androstenedione secretion (Fig. 8.1-3). The output of cortisol and androstenedione (d62/72; Fig 8.2.A and B, respectively, and d62/82; Fig. 8.3.A and B, respectively) were not changed between C and TP cohorts. However, unlike the maternal TP-treated cohort, the peripheral concentrations of testosterone were not significantly different after stimulation in those animals that were fetally androgenised at either d62 and 72 (Fig. 8.2.C), or at d62 and 82 (Fig.8.3.C), compared to controls. Therefore these data suggest that like the adult thecal cell, adrenal androgen synthesis may be enhanced by fetal exposure to maternal androgenisation.

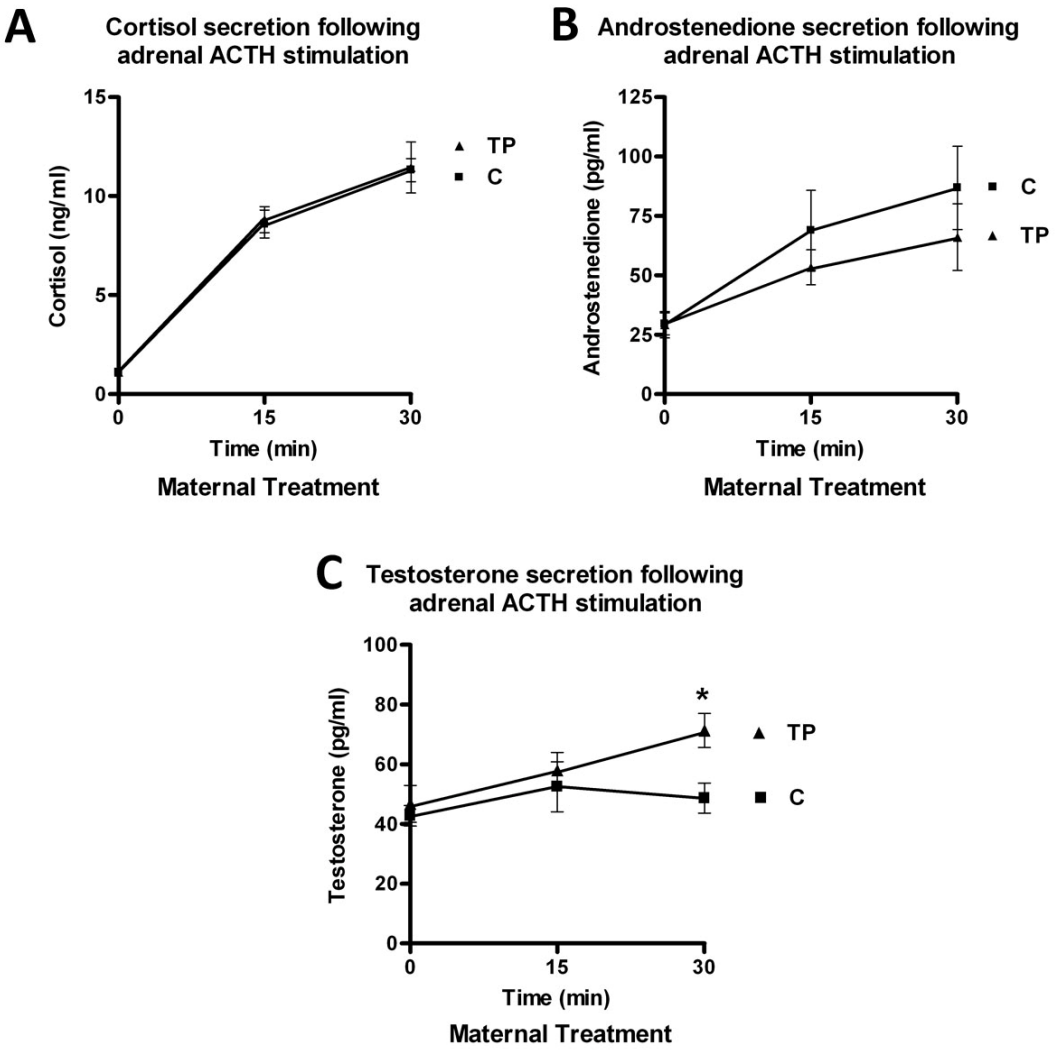

Figure 8.1 The hormonal secretion following adrenal stimulation in maternally androgenised adult sheep. Blood was sampled at a basal time point (0 min) and 15 and 30 min post-ACTH stimulation. Cortisol (A), androstenedione (B) and testosterone (C) were measured by RIA. * $P<0.05$.

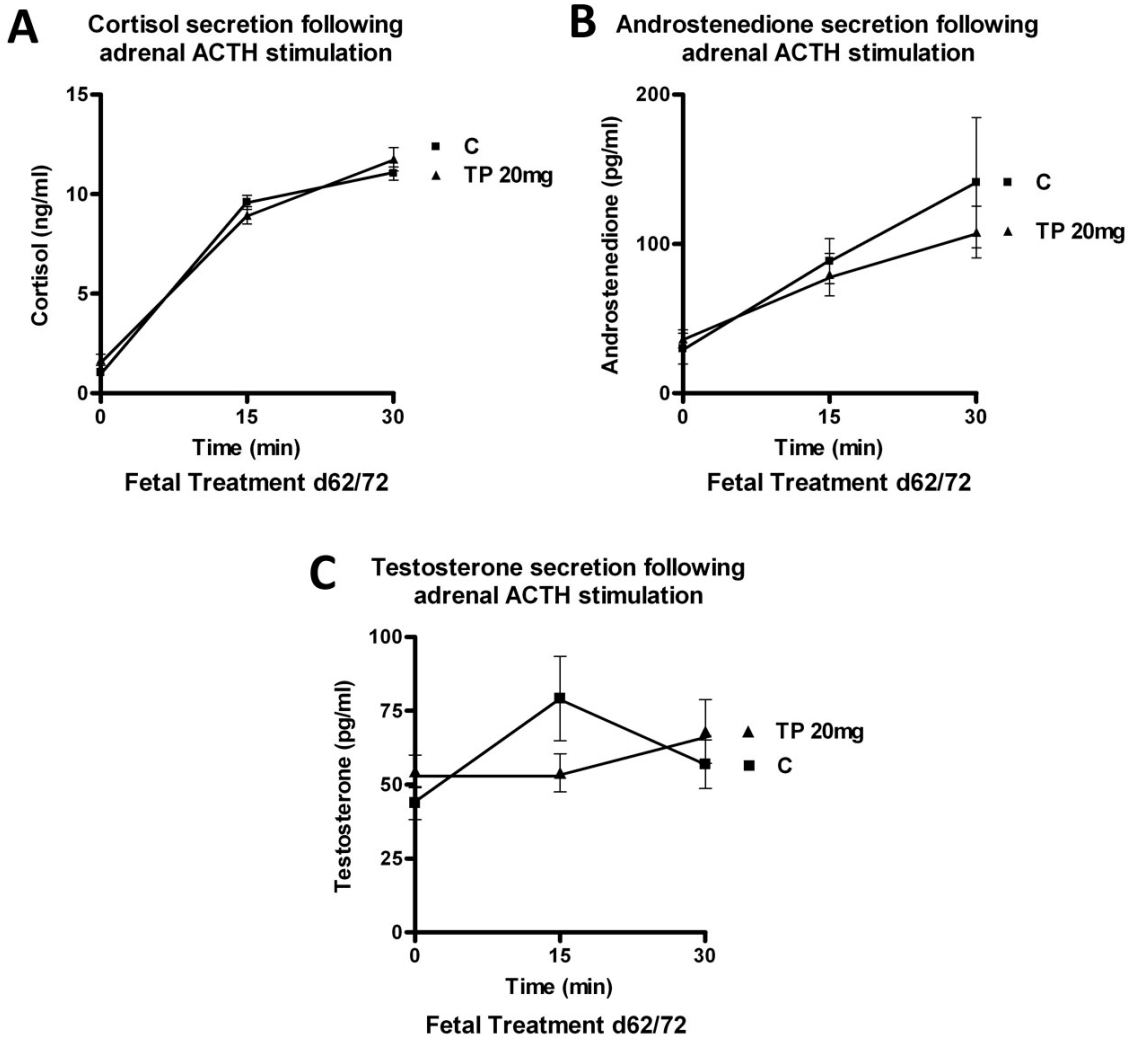

Figure 8.2 The hormonal secretion following adrenal stimulation in fetally d62/72 androgenised adult sheep. Blood was sampled at a basal time point (0 min) and 15 and 30 min post-ACTH stimulation. Cortisol (A), androstenedione (B) and testosterone (C) were measured by RIA.

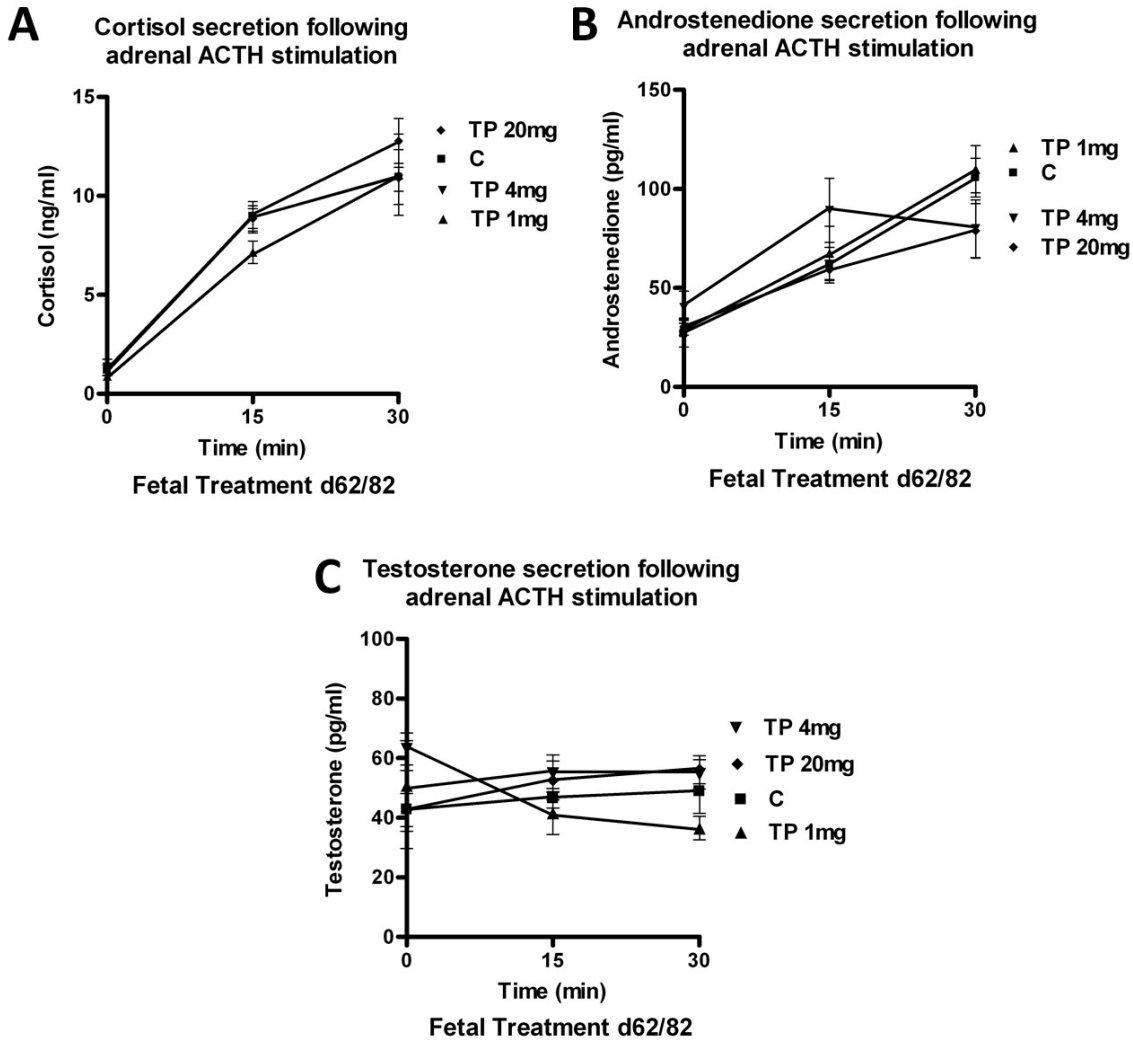

Figure 8.3 The hormonal secretion following adrenal stimulation in fetally d62/82 androgenised adult sheep. Blood was sampled at a basal time point (0 min) and 15 and 30 min post-ACTH stimulation. Cortisol (A), androstenedione (B) and testosterone (C) were measured by RIA.

8.3.2 Expression of steroidogenic enzymes in the adult adrenal

The adrenal gene expression in the adult sheep was studied to detect any functional molecular alterations that may alter the steroid biosynthesis as a consequence of *in utero* exposure to androgens. Adrenal gene expression was studied in the maternal and fetal treated cohorts with regard to *StAR*, *CYP11A*, *HSD3B1*, *CYP17*, *CYP21* and *CYP11B1* expression. The expression of steroid receptors in the adrenal, including *AR* and *ERα*, was also assessed. In addition, to determine whether adrenal gene expression might be regulated by changes in ACTH, the expression of pro-opiomelanocortin (*POMC*), the gene pre-cursor of ACTH, was measured in the adult pituitary.

Maternal androgenisation resulted in alterations in the expression of steroidogenic genes in the adult adrenal. The rate-limiting enzymes of the steroid biosynthesis, *StAR* and *CYP11A* were both up-regulated as a result of maternal TP ($P<0.05$; Fig. 8.4.A and B, respectively). The intermediaries, *HSD3B1* and *CYP17* were also increased in this cohort ($P<0.05$; Fig. 8.4.C and D, respectively). Additionally, the enzymes involved in the glucocorticoid pathway were altered with *CYP21* expression showing a non-significant increase (Fig. 8.4.E) and *CYP11B1* was significantly up-regulated ($P<0.01$; Fig. 8.4.F). These data reveal a mechanism for increased testosterone synthesis in the stimulated adult adrenal from maternally androgenised offspring. Furthermore, the increased adrenal steroidogenic gene expression changes are parallel with the findings observed in the adult thecal cell. In agreement with the results of the synacthen test, there was no effect of fetal androgenisation on the steroidogenic gene expression in the adult adrenal for either fetal treatment cohort (*StAR*, *CYP11A*, *HSD3B1*, *CYP17*, *CYP21* and *CYP11B1*; d62/72 Fig. 8.5.A-F and d62/82 Fig. 8.6.A-F, respectively).

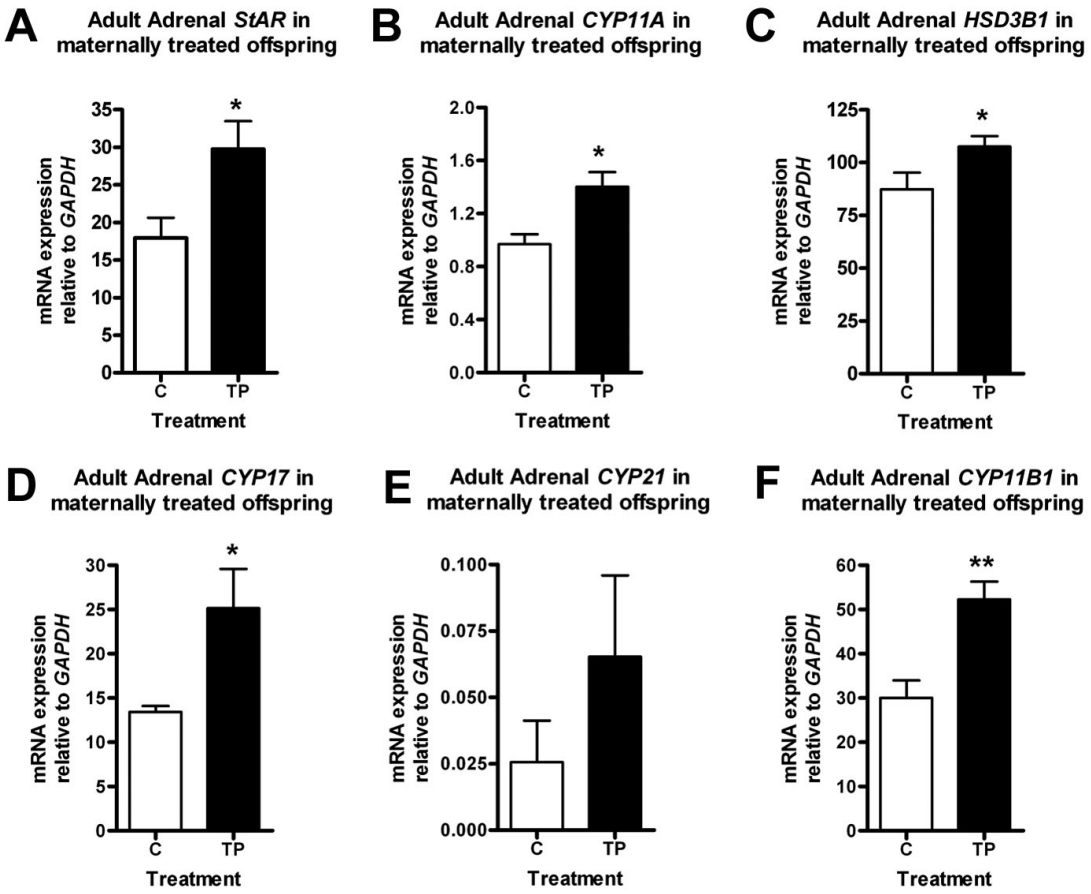

Figure 8.4 The steroidogenic gene expression in the adrenal of the maternally androgenised adult sheep. Genes analysed were *StAR* (A), *CYP11A* (B), *HSD3B1* (C), *CYP17* (D), *CYP21* (E) and *CYP11B1* (F). * $P<0.05$ and ** $P<0.01$.

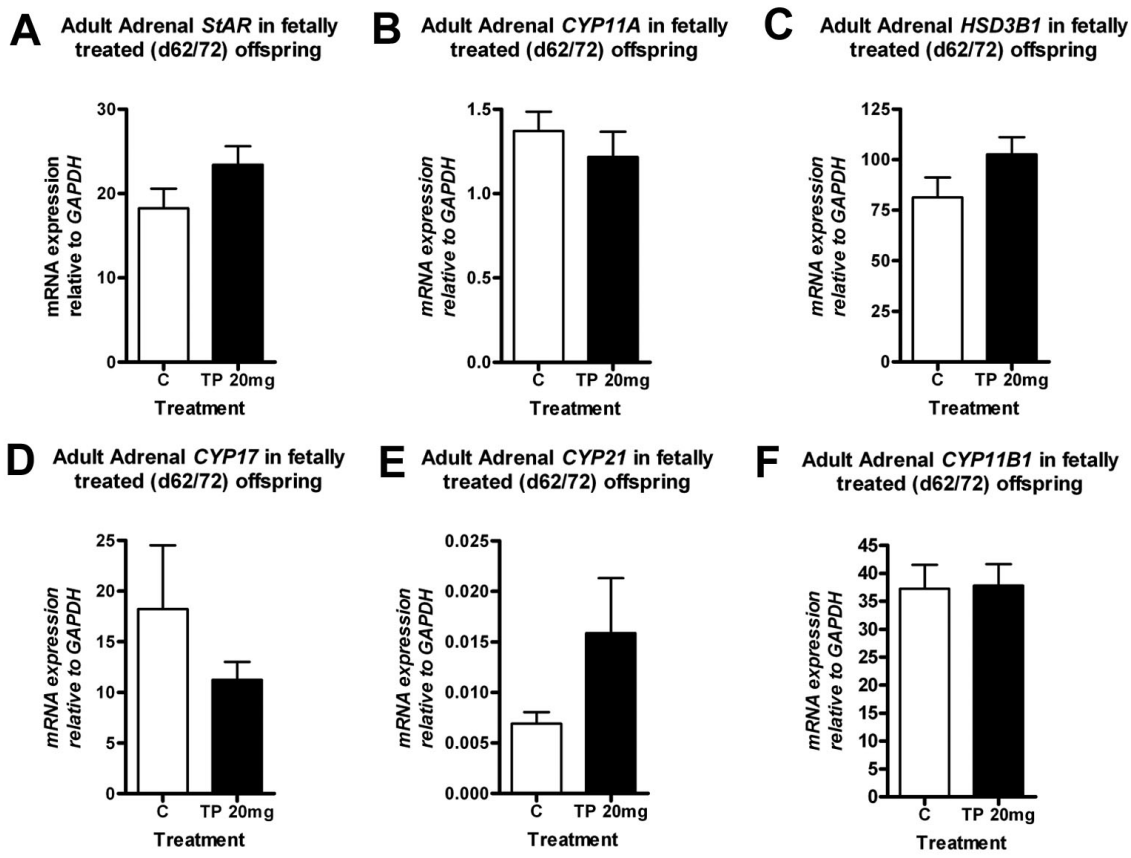

Figure 8.5 The steroidogenic gene expression in the adrenal of the fetally d62/72 androgenised adult sheep. Genes analysed were *StAR* (A), *CYP11A* (B), *HSD3B1* (C), *CYP17* (D), *CYP21* (E) and *CYP11B1* (F).

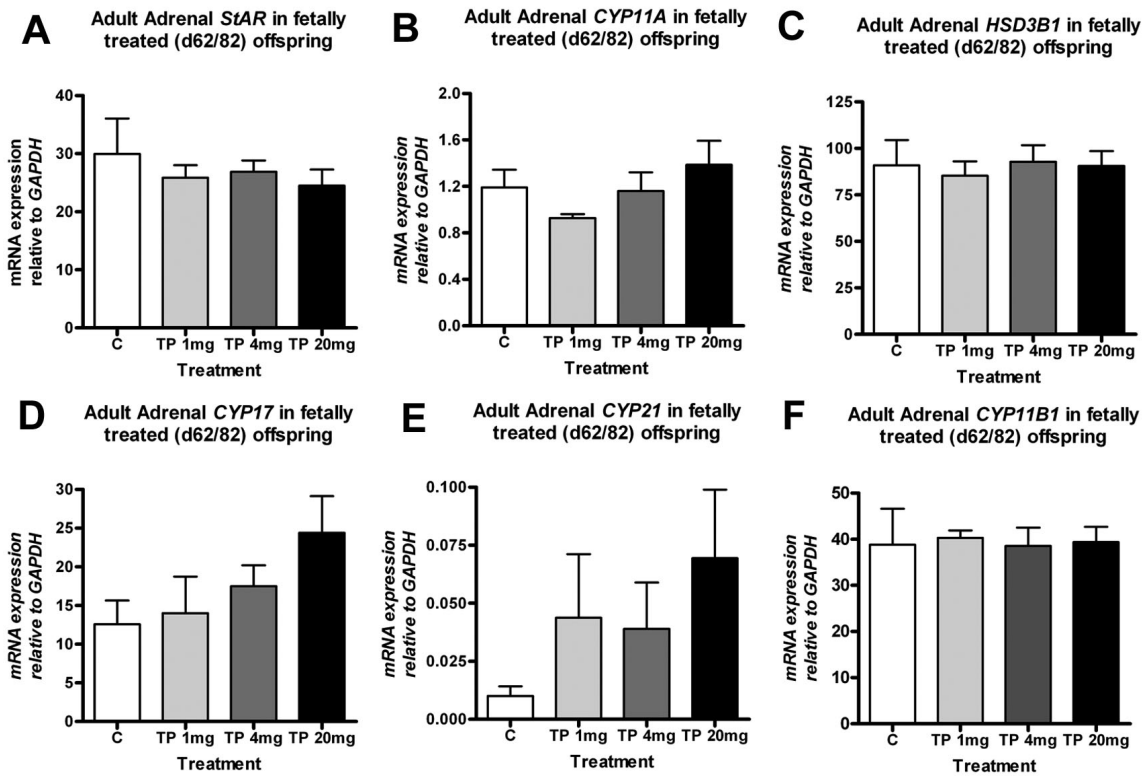

Figure 8.6 The steroidogenic gene expression in the adrenal of the fetally d62/82 androgenised adult sheep. Genes analysed were *StAR* (A), *CYP11A* (B), *HSD3B1* (C), *CYP17* (D), *CYP21* (E) and *CYP11B1* (F).

Additional genes that were studied included the receptors for androgens and estrogens. Maternal androgenisation did not effect the expression of *AR* (Fig. 8.7.A) or *ERα* (Fig. 8.7.B). In the fetally injected cohorts there was an effect of early TP injection (d62/72) on *AR* which was up-regulated (Fig. 8.7.D), however, *ERα* was unaffected (Fig. 8.7.E). Fetal injection at d62 and d82 had no effect on *AR* (Fig. 8.7.G) or *ERα* (Fig. 8.7.H) expression. To assess whether the increase in adrenal steroidogenic gene expression in the maternally treated cohort could be linked to stimulation by pituitary hormones, *POMC* was measured. There was no change in *POMC* gene expression in the adult pituitary (Fig. 8.7.C) suggesting that increased ACTH is not responsible for the increased adrenal gene expression in these animals. Similarly, the expression of pituitary *POMC* was not altered in either fetally TP treated adult cohorts (Fig. 8.7.F and Fig. 8.7.I). Finally, the route of treatment did not alter the expression of these sets of genes in the adrenal or pituitary when the control cohorts were compared (Figs. 8.4-7).

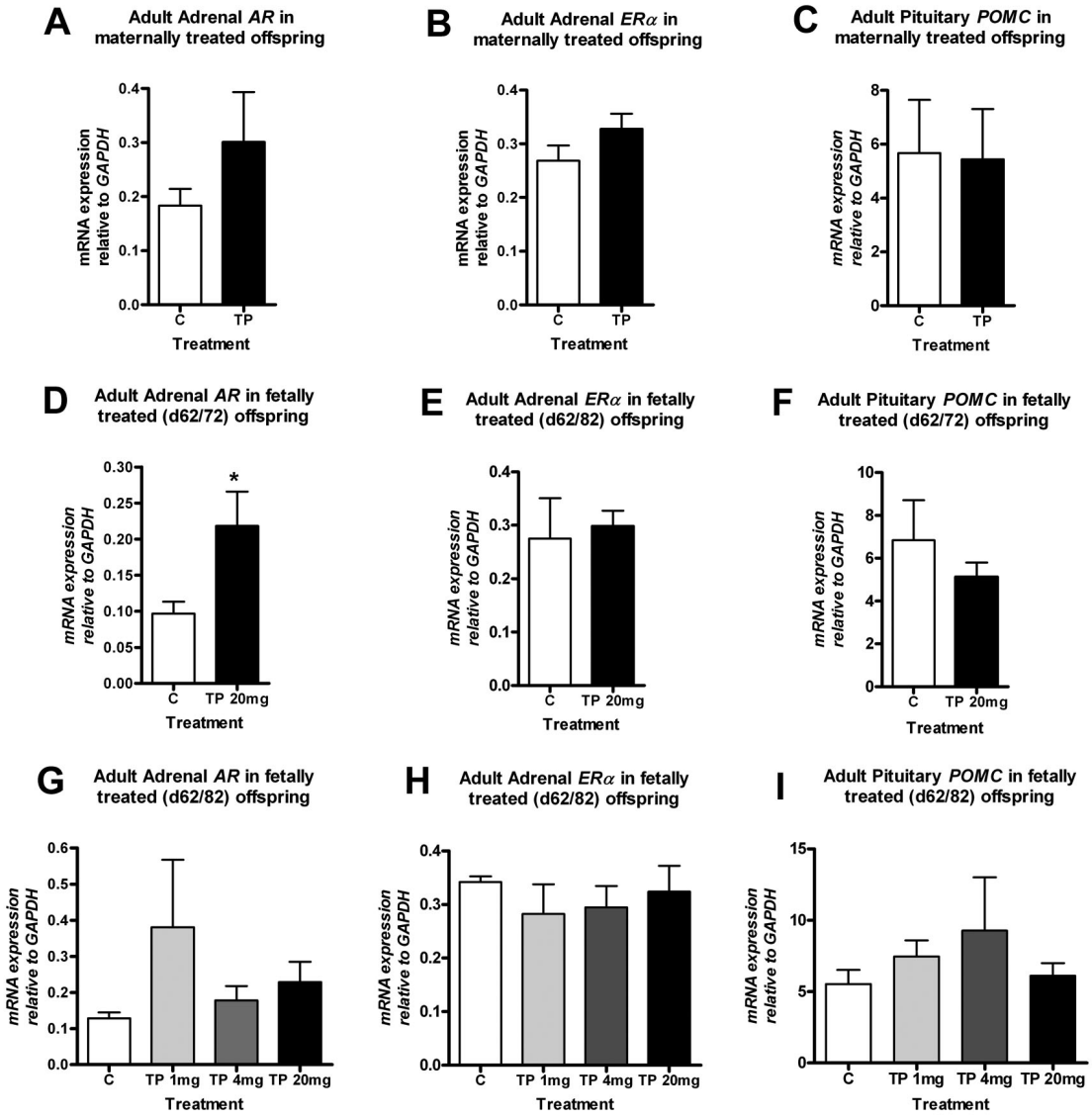

Figure 8.7 Other gene expression in the adrenal and pituitary of the prenatally androgenised adult sheep. Respectively, genes analysed were *AR*, ERα and POMC in the maternally (A-C), fetal d62/72 (D-F) and fetal d62/82 (G-I) treatment cohorts. * $P<0.05$

8.3.3 The effect of maternal prenatal androgenisation in the lamb adrenal

The maternal administration of androgens to the mid-gestation fetus results in increased stimulated adrenal testosterone synthesis in the adult, and there is a global enhancement of steroidogenic genes that are likely to play a role in this. To investigate the developmental period in which this phenotype might arise, adrenal function and gene expression was also

assessed at 10-12 weeks of age (Fig. 8.8). While no significant changes were observed at 10-12 weeks in female maternally androgenised offspring, there were trends that might indicate early effects that would develop into the changes observed in the adult cohort. Testosterone secretion following adrenal stimulation was variable, and tended to be increased in the TP-exposed group (Fig. 8.8.A), although not reaching statistical significance. Likewise, the suggestion of small increases in the lamb adrenal expression of *StAR* (Fig. 8.8.B), *CYP11A* (Fig. 8.8.C) and *CYP17* mRNA (Fig. 8.8.E), did not reach statistical significance. The expression of *HSD3B1* in the lamb adrenal gland (Fig. 8.8.D), was clearly not altered by maternal testosterone administration.

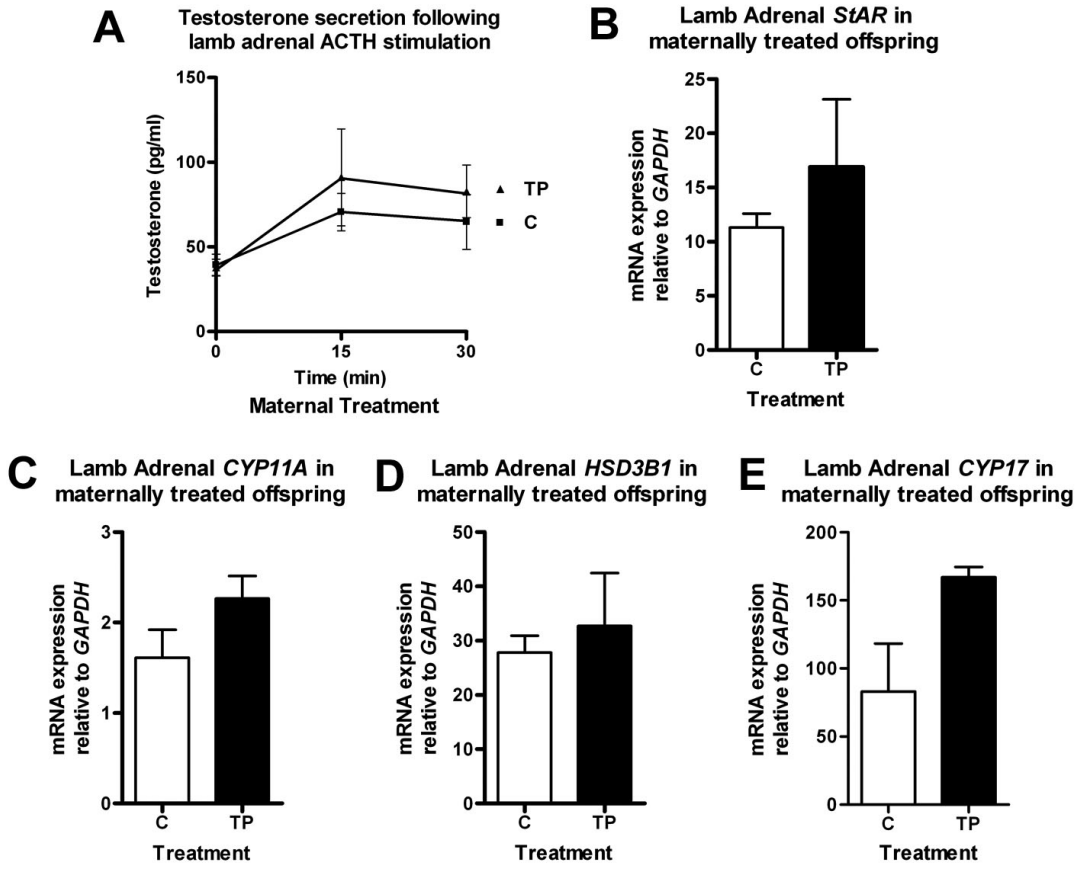

Figure 8.8 Adrenal function in the maternally androgenised lamb. Testosterone was measured by RIA at a basal time point (0 min) and 15 and 30 min post-ACTH stimulation (A). Steroidogenic gene expression was measured in the lamb adrenal including *StAR* (B), *CYP11A* (C), *HSD3B1* (D) and *CYP17* (E).

8.3.4 Fetal adrenal function in the prenatally androgenised sheep model

Since there were changes present in adult adrenal gene expression, and possible subtle alterations in the lamb, in androgenised offspring, the expression of steroidogenic genes was also assessed in the fetal adrenal to determine if there are functional programming effects during development. Adrenal *StAR*, *CYP11A*, *HSD3B1*, *CYP17*, *CYP21* and *CYP11B1* mRNA expression was studied. There was no effect of maternal TP exposure on the fetal adrenal expression of *StAR* by d70 or d90 of gestation (Fig. 8.9.A). *CYP17* tended to rise (not-significant; Fig. 8.9.D) and *CYP21* ($P<0.05$; Fig. 8.9.E) increased with gestational age but neither was affected by maternal androgenisation. In contrast, *CYP11A* ($P<0.05$; Fig. 8.9.B) and *HSD3B1* ($P<0.01$; Fig. 8.9.C) were both significantly up-regulated after 30 days of TP exposure in the d90 fetal adrenal. The expression of *CYP11B1* on the other hand was not altered by gestation or treatment (Fig. 8.9.F).

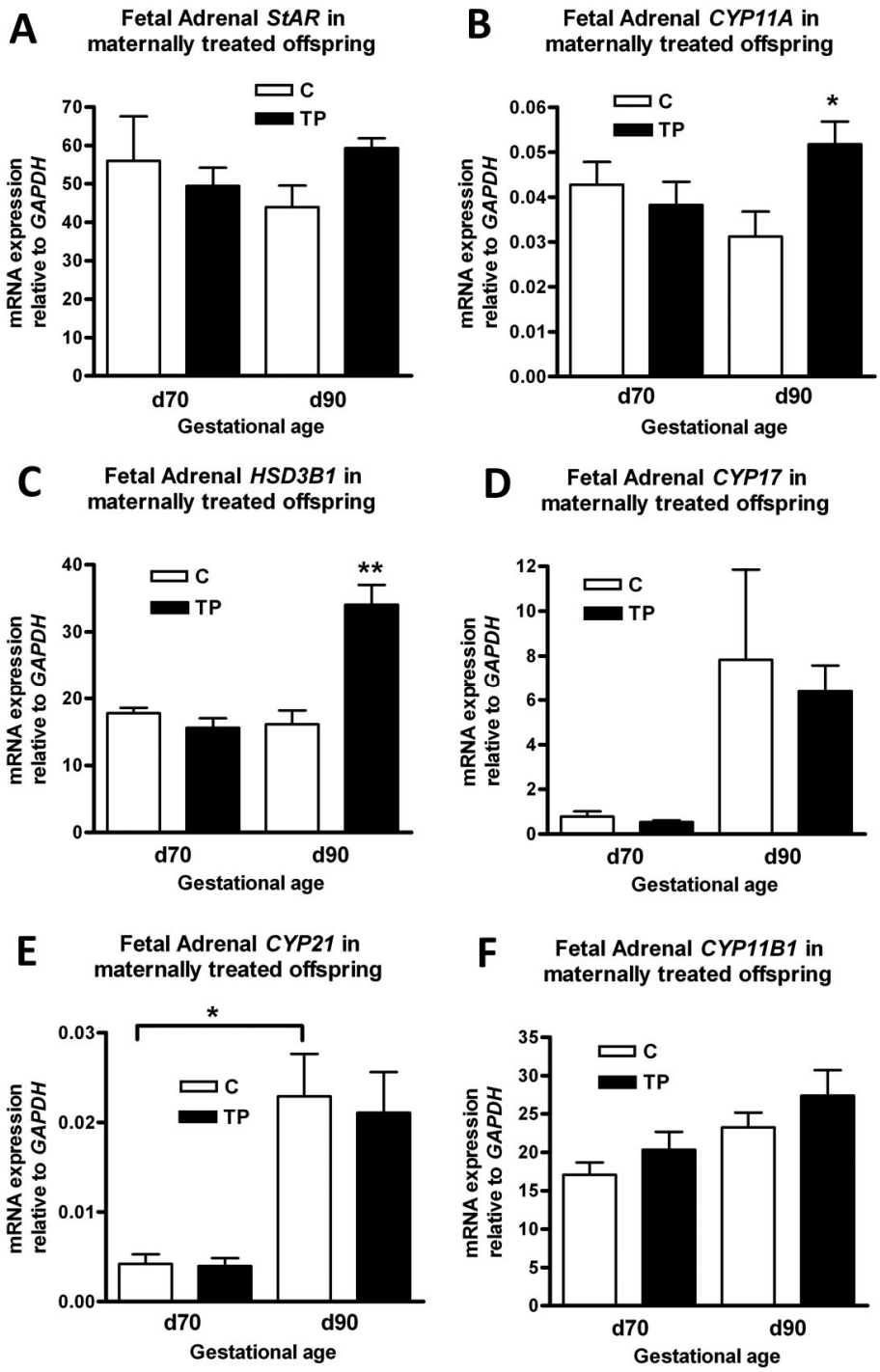

Figure 8.9 The steroidogenic gene expression in the adrenal of the maternally androgenised fetus at d70 and d90. Genes analysed were *StAR* (A), *CYP11A* (B), *HSD3B1* (C), CYP17 (D), *CYP21* (E) and *CYP11B1* (F). * P<0.05 and ** P<0.01.

The effect of direct fetal exposure on fetal adrenal gene expression was also assessed. In contrast to maternal androgenisation, in the fetal injected cohort that had received one injection of TP (20mg) on d60, *CYP17* was significantly up-regulated ($P<0.05$; Fig. 8.10.D) and *CYP21* showed an increased trend (Fig. 8.10.E). There was no effect of fetal androgenisation on the expression of adrenal *StAR*, *CYP11A*, *HSD3B1* or *CYP11B1* (Fig. 8.10.A, B, C and F, respectively) in the d70 fetus.

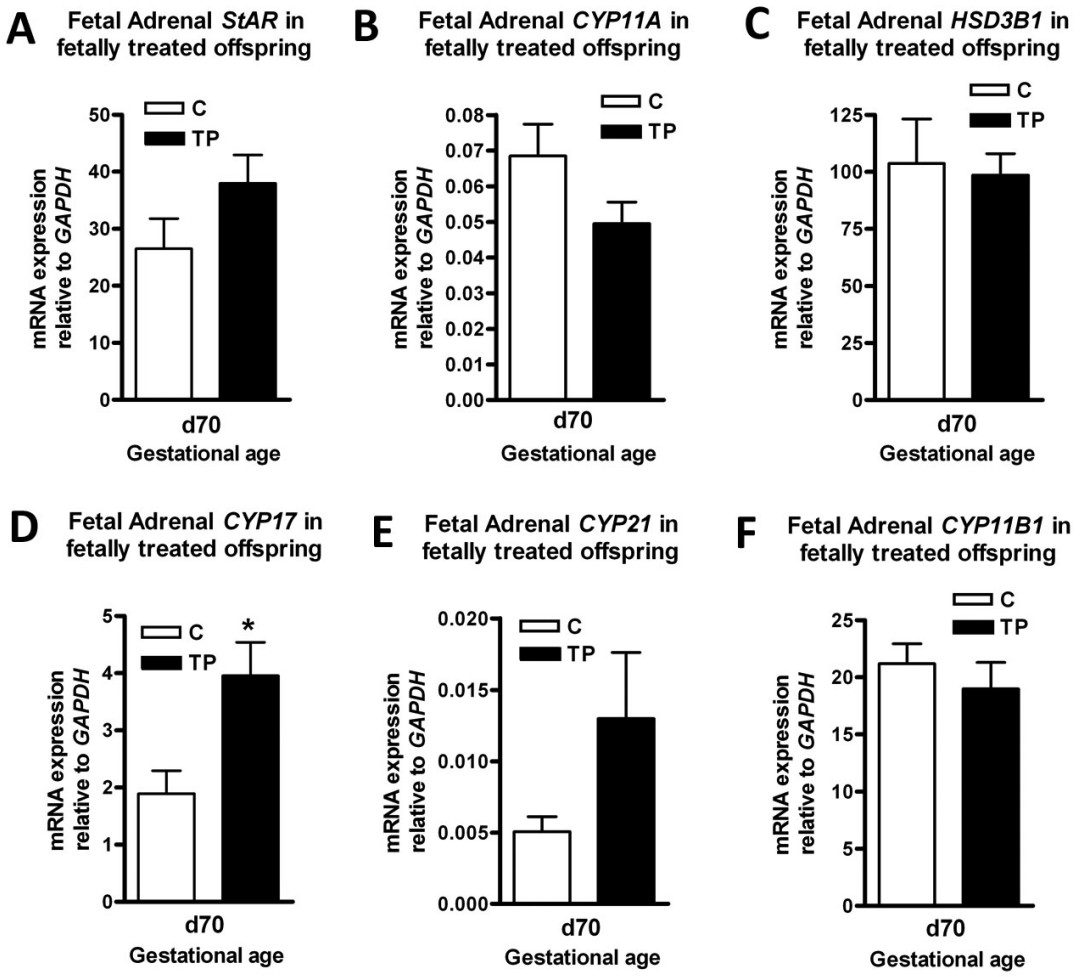

Figure 8.10 The steroidogenic gene expression in the adrenal of the fetally androgenised fetus at d70. Genes analysed were *StAR* (A), *CYP11A* (B), *HSD3B1* (C), CYP17 (D), *CYP21* (E) and *CYP11B1* (F). * P<0.05.

Androgens can directly target the ovine fetal adrenal during mid-gestation (Fig. 8.11). Between gestational d70 and 90 the expression of *AR* tended to decrease (white bars, *P*=0.06; Fig. 8.7.A). Maternal TP exposure for ten days down-regulated adrenal *AR* in the d70 fetus, to that found at d90 (*P*<0.01; Fig. 8.11.A). No effects were found at d90 due to androgenisation. It is possible that the down-regulation of androgen receptivity and likely activity at d70, results in the changes in expression of the steroidogenic genes, observed at d90. Directly injecting the fetus with TP had no effect on the expression of adrenal *AR* by d70 (Fig. 8.11.B). However, comparison of the d70 maternally and fetally treated control cohorts revealed that there was a significant down-regulation of *AR* in the fetally injected control group (*P*<0.01; Fig. 8.11.C). In fact, when similar comparisons are made between steroidogenic gene expression, down-regulated *AR* in the fetal injection group is also associated with a non-significant increase in *CYP17* (*P*=0.13) and down-regulation of *HSD3B1* (*P*<0.05), when compared to the maternal control cohort (Figs. 8.9 and 8.10). These data imply that altered androgen signalling may be associated with changes in the steroidogenic potential of the fetal adrenal gland.

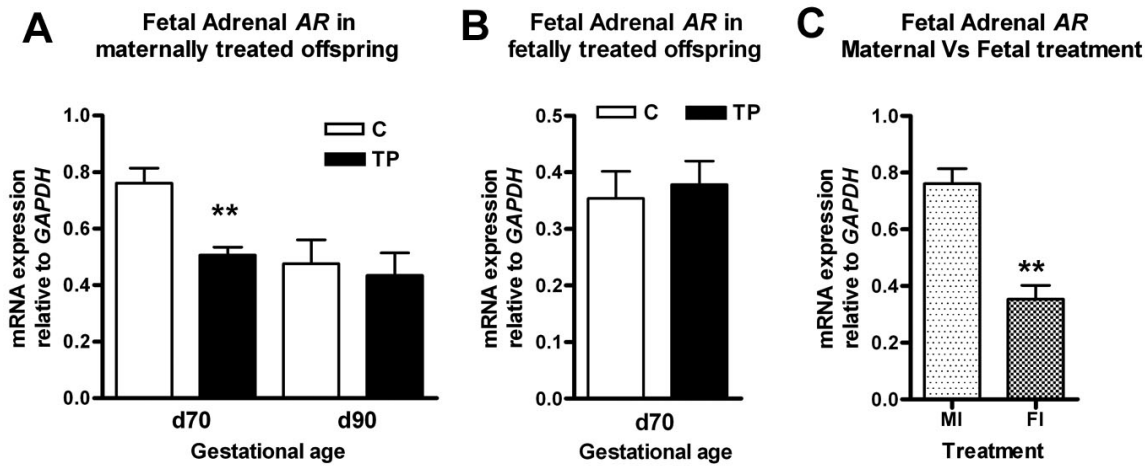

Figure 8.11 *AR* gene expression in the prenatally androgenised fetus. *AR* was measured in the maternally androgenised fetus at d70 and d90 (A) and in the fetally androgenised fetus at d70 (B). ** *P*<0.01.

8.4 Discussion

The effect of prenatal androgens in the developing adrenal, the pre-pubertal lamb, and adult were assessed to ascertain whether there was a programming effect of androgens or estrogens that were identifiable in an animal model in which the peripheral hormonal environment was normal, and to distinguish at what time-point any changes are observable. There was a clear effect of prenatal androgens in the adult adrenal as a consequence of maternal TP exposure. While androstenedione secretion was not enhanced following ACTH stimulation in the maternally TP-exposed cohort, serum testosterone concentration was significantly increased in this group 30 min post-ACTH. In addition, gene expression analysis revealed the up-regulation of *StAR*, *CYP11A*, *CYP17* and *HSD3B1* that might shunt steroid precursors in the direction of androgen biosynthesis. In the absence of changes in basal or ACTH-stimulated cortisol secretion, adrenal *CYP11B1* gene expression was significantly up-regulated in the maternally TP-treated animals compared to controls. CYP11B1 is required for the final step in the glucocorticoid biosynthesis pathway converting deoxycortisol into cortisol (134). Peripheral cortisol concentration was not altered in these animals; nevertheless, it may be that the increase in *CYP11B1* is not sufficient to drive increased cortisol production in the absence of increased substrate. Unlike the changes observed in the maternally treated cohort, there did not appear to be any alteration in adult adrenal function in the fetally treated cohort in response to either the fetal injection or to TP exposure.

The effects of maternal TP in the adult adrenal may be mediated through the actions of estrogens that reach the fetus via placental aromatisation (377), since long-term effects of direct fetal TP on adrenal hormonal production and steroidogenic gene expression were not observed. In these cohorts basal concentrations of circulating androgens, testosterone and androstenedione and the estrogen, estradiol, were not altered in any of the treatment groups (Chapter 6) implying that the changes in the adrenal are not affected by an altered sex steroid environment. With the exception of the d62/72 fetally TP-treated cohort, the expression of adrenal *AR* and *ERα* mRNA was also not affected by prenatal androgenisation suggesting that the alterations in steroidogenic signalling are independent of androgen or estrogen signalling in the adult adrenal. In addition, there was no change in the amount of *POMC* expressed by the pituitary, indicating that the alterations in steroidogenic gene expression are likely intrinsic and not a result of altered pituitary ACTH stimulation. The consequence of

increased *AR* gene expression in the d62/72 fetal TP cohort is not known, and no other genes in this group were altered.

To investigate whether the adult adrenal phenotype that was observed in the maternally androgenised cohort might be a result of long-term changes in the adrenal, or, progressively develops as the animal matures, adrenal function in the 10-12 week lamb was also assessed. While no definitive changes in function were observed, there did appear to be an increased propensity for enhanced steroidogenesis that could translate into the significant alterations in the adult adrenal, suggesting that there were programming effects inherent to this tissue.

Subsequent examination of the fetal adrenal did identify alterations in steroidogenic gene expression during the time of testosterone exposure. In maternally TP exposed fetuses, by d90, but not d70, the expression of *CYP11A* and *HSD3B1* was increased; however, exposure to increased androgens did not effect the expression of other steroidogenic genes. In the control maternal cohorts, *AR* showed a non-significant trend for down-regulation between gestational d70 and d90. At d70, the maternal exposure of the fetus to androgens for ten days led to the down-regulation of adrenal *AR* mRNA to the same level observed in the d90 control cohort. At d90 prenatal androgens had no effect on *AR* expression. It is possible that increased circulating levels of androgens resulted in the down-regulation of adrenal *AR* by d70 to act in a negative manner to dampen further the adrenal androgen output. While the steroidogenic genes are not affected at d70, by d90 the increase in *CYP11A* and *HSD3B1* may reflect a programming affect of a moderate loss in adrenal androgen signalling. It is also possible that estrogens may have a role in regulating AR expression at this time since there was no change in *AR* mRNA in the adrenals of animals directly exposed to TP. It was noted however that the expression of *AR* was down-regulated as a result of the fetal injection, suggesting that alternative programming pathways may be at play.

In the fetus, the adrenal gland produces cortisol in a triphasic pattern over gestation. In this study the assessment of the fetal adrenal coincided with the gestational period in which the adrenals are producing steroids before cortisol secretion declines from around d90-120 (604). Interestingly the levels of *CYP17* and *CYP21* were increased at d90 compared to d70 in control animals which does not marry very well with the fact that adrenal steroidogenesis declines at this stage, however these genes may increase throughout development and it is the drop in ACTH from d90-120 that leads to a transient down-regulation in these enzymes

after d90. In this regard, Tangalakis *et al.* demonstrated a direct relationship with ACTH stimulation and *CYP17* gene expression although they did not find this relationship with the expression of *CYP21* (603).

In the fetal TP injected cohort *CYP17* was significantly up-regulated in the d70 adrenal which represented a 2-fold increase from fetal injection controls and a 4-fold increase from maternal controls. Also while *HSD3B1* mRNA expression in the d70 adrenal of fetally TP exposed animals was not different to controls, it was significantly down-regulated compared to maternal controls. The overall adrenal phenotype of the TP-exposed fetally injected fetus is therefore a combination of lowered *AR* that is associated with an increase in *CYP17* and a decrease of *HSD3B1*. Speculatively this may suggest that the loss of androgen signalling in this model could result in the modulation of the expression of steroidogenic genes that might favour the androgenic biosynthesis pathway.

In conclusion, both the adult and fetal adrenal glands are affected by prenatal androgenisation, with possible subtle differences observed in the lamb. It appears that the changes in the fetal adrenal, in response to maternal and fetal TP exposure, may occur via independent pathways and have alternative long-term effects. The phenotype observed in the fetally injected control and TP d70 adrenals may be transient as there was no adult adrenal phenotype present in these animals with regards to the parameters assessed. The long-term effects of the d70 fetally injected adrenal phenotype are not known, but there may be a role for the programming of other systems in the fetus through the androgenic actions of TP and/or elevated levels of cortisol (Chapter 3). On the other hand, maternal TP-exposure, possibly through the programming actions of estrogen, may exert developmental effects on the fetal adrenal that lead to the changes in the adult adrenal.

In the adult prenatally androgenised sheep, not only is there an enhanced drive for the ovary to secrete androgens, but steroidogenic gene expression is increased in the adrenal, which can also excessively produce testosterone when stimulated *in vivo*. This occurs in the absence of an altered hormonal environment such as LH and androgens. It is however possible that these changes are affected by alternative factors that are present in the adult, which include metabolic function, and that such features may contribute the development of an ovarian and/or adrenal phenotype that is not present in younger animals. The following

chapter investigates the effect of prenatal androgenisation on metabolic homeostasis and liver function in the adult sheep.

Chapter 9

The effect of prenatal androgens on metabolic parameters

9.1 Introduction

An abnormal or altered fetal environment is emerging as a likely aetiological factor for a host of adult diseases (274, 517, 606). The maternal environment can influence epigenetic processes in the placenta and fetus that programme lasting developmental changes associated with cardiovascular disease, hypertension, obesity and type II diabetes (517, 519, 606, 607), all of which are prevalent in Westernised societies (428, 429). Women with PCOS often present with these metabolic perturbances in addition to the reproductive and endocrine characteristics of the syndrome (166). More than one third of obese women with PCOS are insulin resistant (167) and these women are more likely to develop type II diabetes than weight-matched controls (449, 450). However, while PCOS women are hyperandrogenaemic (162), and androgens are linked with the development of central obesity and insulin resistance (608, 609) some women with PCOS are lean and have normal BMIs. These women do however retain the hyperinsulinaemic feature of PCOS regardless of their weight, indicating a pancreatic defect that is likely augmented by obesity (434, 452). Metabolic parameters such as hyperinsulinaemia, insulin resistance and increased adiposity are also reported in animal models of PCOS in which the fetus is exposed to elevated androgens (298, 365, 477-479).

In this chapter the metabolic effects of prenatal androgenisation of the ovine fetus were assessed with particular focus on the liver. Women with PCOS are more likely to have abnormal liver function tests (610, 611) including raised aminotransferases (alanine aminotransferase [ALT] and aspartate aminotransferase [AST]). Elevated aminotransferases are linked with obesity, increased triglycerides, insulin resistance and are often an indicator of non-alcoholic fatty liver disease (NAFLD) (611-613), which has also been associated with PCOS (614-616). NAFLD occurs through liver damage leading to steatosis and in severe cases hepatic inflammation, liver fibrosis or cirrhosis (613). The metabolic features of PCOS therefore seem to involve a complex interplay between increased androgen concentrations, insulin signalling, central obesity and NAFLD (162, 168).

An assessment of NAFLD in young adult ewes exposed to increased androgen concentrations *in utero,* and investigation of the metabolic and synthetic function of the liver at a molecular level, was carried out. The relationship between findings in the liver, insulin

resistance and central obesity was determined to provide novel insights into possible early hepatic alterations associated with a PCOS-like condition. The aims of this chapter were to establish: A) whether prenatal androgenisation is associated with the development of NAFLD or clinical determinants of liver damage. This included measurements of ALT and AST as well as other analytes including alkaline phosphatase (ALP) and gamma glutamyltransferase (GGT) of which elevated levels would indicate liver dysfunction such as bile duct blockage (617). B) If *in utero* exposure to androgens is linked to the development of central obesity by the measurement of omental fat as well as other metabolic parameters including plasma triglycerides, leptin and cholesterol concentrations. C) Whether treatment results in a metabolic phenotype affecting glucose and insulin homeostasis as determined by glucose tolerance testing. The effect of prenatal androgenisation on adult liver function was investigated by examining D) the expression of hepatic steroid receptors, E) liver metabolic activity, by assessing expression of the gluconeogenic enzyme PEPCK, and F) growth factor secretion by analysing the hepatic expression of IGF1. These experiments were conducted in animals that were maternally exposed to androgens from d62-102 of gestation and those that were fetally injected with TP at d62 and d82. A final aim of this chapter was to characterise the effects of these differential treatments on the liver and metabolic outcome of these androgenised offspring.

9.2 Materials and Methods

9.2.1 Experimental animals

The female animals assessed in this chapter are detailed in Table 9.1.

Study	Treatment	Sample number (n)
Adult		
Fetal Treatment	Injection at d62 and 82	C = 4, TP 20mg = 7
Maternal Treatment	Treatment from d62-102	C = 5, TP = 9

Table 9.1 The experimental animals included in the assessment of metabolic parameters, treatment regime and corresponding sample numbers.

Animals were fasted and a GTT performed on the day of sacrifice. Bolus glucose (500mg/ml in 20ml) was administered intravenously following collection of a peripheral basal blood sample. Blood was re-sampled after 15 min and the animal immediately euthanised and tissue collected. Equivalent liver samples were snap frozen and stored at -80°C, or embedded in cassettes containing OCT compound (VWR International) and snap frozen. Alternatively tissue was stored in Bouins fixative for 24 h, transferred to 70% ethanol and embedded in paraffin wax. In addition, the omental fat was removed and weighed. Blood was decanted into a heparinised test-tube for hormonal/metabolite measurements or S-Monovettes containing sodium fluoride combined with anti-coagulant (Sarstedt Ltd, Nümbrecht, Germany) for glucose measurement. Tubes were centrifuged at 3000 rpm for 15 min at 4°C and the plasma collected and stored at -20°C.

9.2.2 Measurement of glucose and insulin

Glucose concentrations were measured with a colourimetric glucose assay kit (Alpha Laboratories Ltd., Eastleigh, UK), using a Cobas Fara centrifugal analyser (Roche Diagnostics Ltd.). Glucose is oxidised by glucose oxidase into gluconic acid and hydrogen peroxide, which, in the presence of peroxidase reacts with 4-aminoantipyrine and hydroxybenzoic acid, forming a red compound. Colour intensity monitored at 500nm is

proportional to the concentration of glucose in the sample. Assay sensitivity was 0.2mmol/l and intra- and inter-assay CVs were <2% and <3%, respectively. Insulin was measured using an ovine insulin ELISA kit (80-INSOV-E01, ALPCO Diagnostics, Salem, NH, USA) as per the manufacturer's instructions. Absorbance was measured colourimetrically on a ThermoMax Microplate Reader (Molecular Devices, CA, USA) at 450nm with a reference wavelength of 650nm. A standard curve comprising a cubic spline fit was generated and insulin concentrations calculated using SoftMax Pro software (Molecular Devices). Assay sensitivity was 5.0pg/ml and intra- and inter-assay CVs were <5% and <6%, respectively[7].

9.2.3 Measurement of other plasma analytes

To measure the concentration of cholesterol, free fatty acids (FFA), triglycerides, ALT, AST, ALP and GGT, commercial assay kits were employed (Alpha Laboratories Ltd., Eastleigh, UK) as per the manufacturer's protocols, using a Cobas Fara centrifugal analyser (Roche Diagnostics Ltd.) to obtain analyte concentration. Assay sensitivities for cholesterol, FFA, triglycerides, ALT, AST, ALP and GGT were 0.1mmol/l, 0.05mmol/l, 0.02mmol/l, 4U/l, 6U/l, 20U/l and 5U/l, respectively, and the overall CVs were all <3%[8]. Serum leptin concentrations were obtained using a Multi-Species Leptin RIA kit with an antibody against human leptin (XL-85K, Millipore, Missouri, USA). Specific leptin RIA details are listed in Appendix A. Assay sensitivity was 1ng/ml and intra- and inter-assay CVs were <4% and <7%, respectively.

9.2.4 Oil Red O Lipid Staining and Analysis

Frozen 5µm sections from OCT embedded liver samples were cut (Leica Cryostat Microtome 1900, Leica Biosystems Nussloch GmbH, Heidelberger, Germany) at -20°C, mounted onto glass slides and air-dried. Tissue was fixed in 10% formalin for 10 min, rinsed in dH_20 and air-dried before a further rinse in 60% isopropanol. Sections were stained with Oil Red O dissolved in 60% isopropanol for 15 min at room temperature and rinsed in 60%

[7] The measurement of plasma glucose and insulin was carried out by Charlotte Wood, under supervision by KH
[8] The measurement of plasma analytes was performed by Dr Forbes Howie.

isopropanol followed by washing in dH_20. Tissue was counterstained in haematoxylin, washed thoroughly in dH_20 and mounted in Aqua-Mount media (Fisher Scientific Ltd.). Unsaturated hydrophobic lipids were identified microscopically by the presence of a red stain and was subsequently quantified blindly by two independent investigators by classification into negative (-), possible early (-/+) and positive (+) fatty liver categories by comparison to pre-agreed reference sections.

9.2.5 Quantitative real time -PCR

The extraction of mRNA from tissues containing a high blood, lipid or fibre content necessitates the use of the TRI-reagent method instead of the on-column method as column blockage would result in low a RNA yield. Adult liver was extracted using the TRI-reagent protocol. This method combines the lysis and purification of samples with guanidine-thiocyanate and that of phenol and chloroform to achieve RNA, DNA and protein isolation. RNA was extracted from a representative ~50mg frozen liver sample. Tissue was homogenised in 1ml TRI-reagent using a Qiagen TissueLyser (Qiagen Ltd.), rested for 5 min and phase separated with the addition of 0.2ml chloroform and vigorous shaking for 15 sec. Samples were incubated for 5 min at room temperature and centrifuged at 12,000 g at 4°C for 20 min. The upper aqueous phase containing RNA was collected, mixed with 0.5ml isopropanol, and incubated for 10 min at room temperature. Samples were centrifuged at 12,000 g at 4°C for 15 min and the RNA pellet washed in 1ml 75% ethanol prior to centrifugation at 7,500 g and 4°C for a further 10 min. The pellet was subsequently air-dried before elution in 50µl nuclease-free water and stored at -80°C. Measurement of RNA concentration, cDNA synthesis and qRT-PCR were performed in adult liver as described in Section 2.8. The primer sequences used for PCR are listed in Table 9.2.

Gene (Accession No.)	Forward Sequence	Reverse Sequence	Product Size (bp)
AR (XM_001253942)	GCCCATCTTTCTGAATGTCC	CAAACACCATAAGCCCCATC	233
ERα (NM_001001443)	GAATCTGCCAAGGAGACTCG	CCTGACAGCTCTTCCTCCTG	187
GR (NM_001114186)	AAGTCATTGAACCCGAGGTG	ATGCCATGAGGAACATCCAT	207
IGF1 (NM_001009774)	CATCCTCCTCGCATCTCTTC	CTCCAGCCTCCTCAGATCAC	239
IGF1R (XM_002696504)	CCAAAACCGAAGCTGAGAAG	TCCGGGTCTGTGATGTTGTA	199
IGFBP1 (NM_001145177)	TCCCCAGAGAGCTCAGAGAT	CTGCTCCCTGGCTAATCTGT	207
PEPCK (NM_174737)	AAAGAGATACGGTGCCCATC	ATGCCAATCTTGGACAGAGG	178
GAPDH (NM_001034034)	GGCGTGAACCACGAGAAGTATAA	AAGCAGGGATGATGTTCTGG	229

Table 9.2 Liver gene analysis using SYBR Green qRT-PCR. Gene names and accession numbers, forward and reverse primer sequences (5'-3') and PCR product sizes are listed.

9.2.6 Immunohistochemistry

A portion of liver was processed for histology using the standard method (Section 2.4.1), and immunohistochemistry (Section 2.4.3.1) was carried out using antibodies to AR and PEPCK. For negative controls an AR-specific blocking peptide was available and for PEPCK immunostaining negative controls consisted of omission of the primary antibody and substitution with non-specific IgGs. Antibody details and optimised working concentrations are listed in Table 9.3.

Antigen	Antibody Clone/Source	Dilution	Secondary antibody
AR	Polyclonal Rabbit (N20; sc-816) Santa Cruz Biotechnology Inc., Santa Cruz, USA	1:100	GARB
PEPCK	Polyclonal Rabbit (H300, sc-32879) Santa Cruz Biotechnology Inc.	1:100	GARB

Table 9.3 List of antibodies used for immunohistochemistry in the adult liver. The antibody suppliers, working concentrations and appropriate secondary antibodies are detailed.

To confirm that the staining for PEPCK in the liver was specific a Western blot was performed (Section 2.7) using adult control liver homogenates. A single band for PEPCK was visualised at 62kDa (Fig. 9.1).

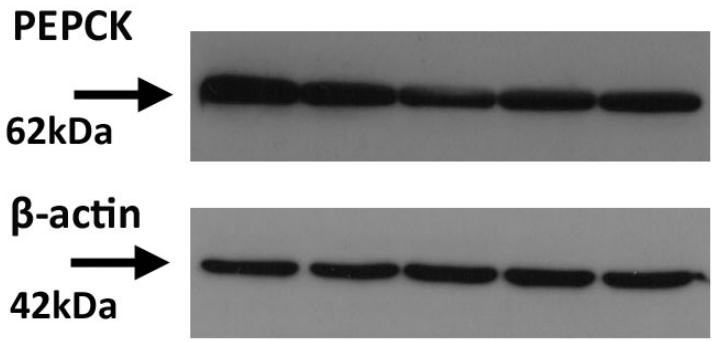

Figure 9.1 Validation of PEPCK immunohistochemical staining specificity by Western blot. In each lane 20µg of protein was loaded. Primary antibodies applied were β-actin as an internal control (1:40,000; AC-15 mouse monoclonal, A5441, Sigma-Aldrich) or PEPCK (1:6000; H-300 rabbit polyclonal, sc-32879, Santa Cruz Biotechnology Inc.).

9.2.7 Statistical analyses

An unpaired Student's t-test or a Mann Whitney non-parametric test was applied when group variances were not equal or the data were not normally distributed, to compare the means of two groups including analyte and hormonal assessment and gene expression analysis. Values are presented as mean ± sem. A chi-squared test was employed when analysing differences between the proportions of animals classified into liver phenotype groups and values are presented as percentages. P values of $P<0.05$ were regarded as significant.

9.3 Results

9.3.1 Maternal prenatal androgenisation is associated with subclinical fatty liver in adults that is not related to central obesity

To determine whether prenatal androgenisation is associated with the development of fatty liver in young adult sheep, liver tissue was examined for signs of lipid accumulation and plasma was analysed for clinical features of liver damage. An Oil red O histological lipid stain was performed on frozen liver sections and staining was classified into negative (-), possible early (+/-), or positive (+) for fatty liver (Fig. 9.2.A), as determined by the presence of red lipid globules. Androgenisation of female fetuses by maternal TP treatment from d62-102 of gestation resulted in the presence of fatty liver in offspring at 11 months of age ($P<0.05$; Fig. 9.2.B). These early signs of liver damage were not however detectable at a clinical level as there was no change in serum determinants of liver function including circulating AST (Fig. 9.2.C), GGT (Fig. 9.2.D), ALT or ALP (Appendix C) concentrations. While fat accumulation was present in the liver, prenatal testosterone exposure did not lead to central obesity (omental fat; Fig. 9.2.E) in adult sheep. These findings were consistent with the lack of change in circulating leptin concentrations (Fig. 9.2.F). Other metabolic parameters including plasma FFA, triglycerides (Appendix C) and cholesterol (Fig. 9.2.G) were not altered.

9.3.2 Insulin homeostasis in maternally treated adult offspring

The presence of fatty liver without central obesity in adults from androgenised mothers indicates early metabolic disruption that might be further manifested systemically. Glucose tolerance tests were carried out just prior to sacrifice to determine pancreatic response to glucose. Following bolus glucose administration, initial glucose dynamics between control and TP-exposed animals were not altered (Fig. 9.2.H), however the insulin response to glucose stimulation was significantly greater in the treatment group ($P<0.05$; Fig. 9.2.I), suggesting a perturbed pancreatic response. Additionally, basal circulating insulin levels were ~3-fold higher in adults maternally exposed to TP (Fig. 9.2.I), though this did not reach statistical significance ($P=0.071$).

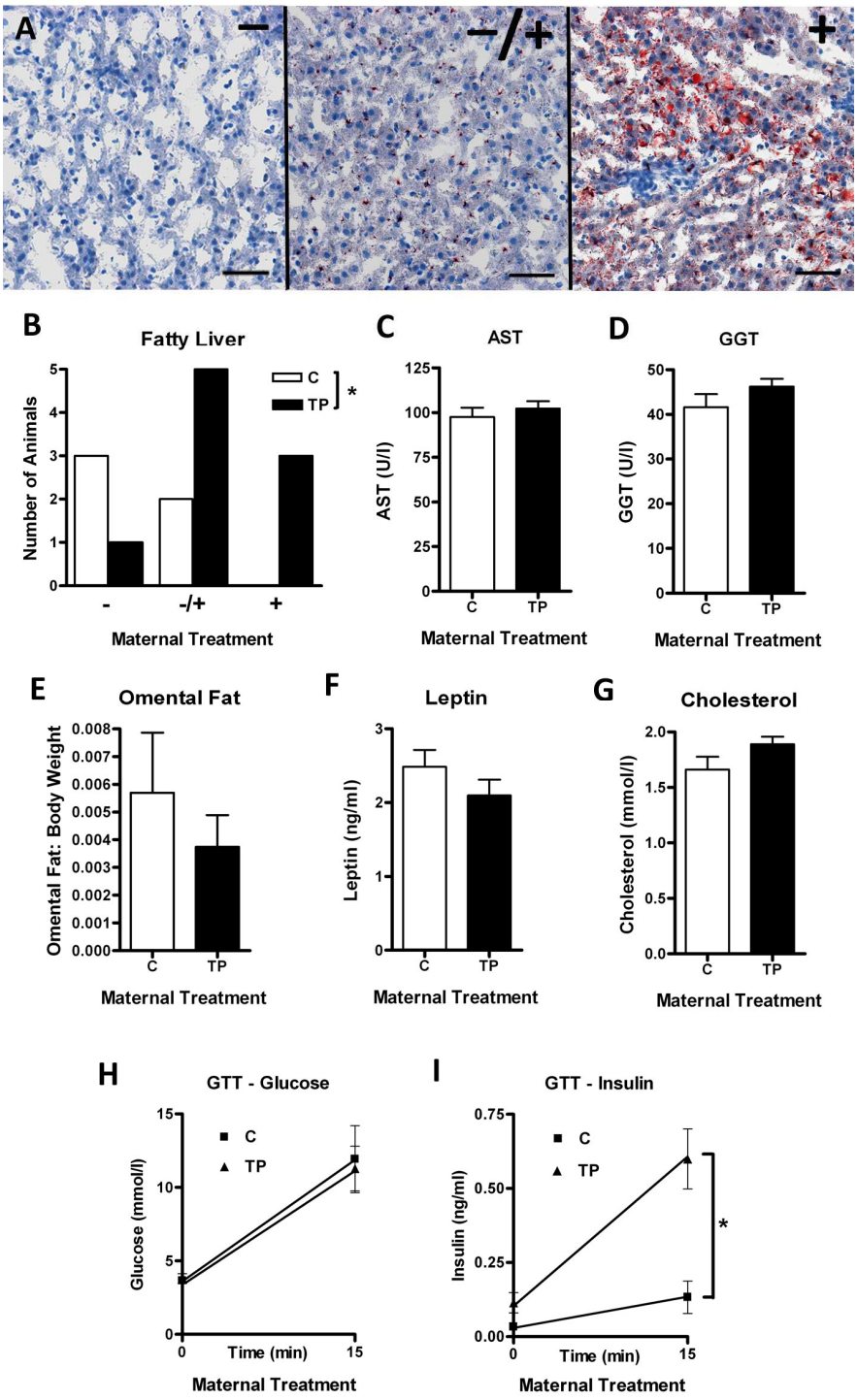

Figure 9.2 The effect of maternal prenatal androgenisation on adult liver lipid content and other metabolic parameters. A) representative Oil red O histological stains for lipids in ovine adult liver sections illustrating negative (-), possible early (+/-) or positive (+) fatty liver (scale bars = 50µm) and B) analysis of fatty liver in C and TP cohorts. Effect of maternal prenatal TP treatment on liver analytes including AST (C) and GGT (D), weight of omental fat (E), plasma leptin (F) and cholesterol levels (G). Plasma glucose (H) and insulin (I) concentrations at basal levels and 15 min after i.v. glucose bolus. * $P<0.05$.

9.3.3 Alterations in the expression of hepatic steroid receptors

As subclinical fatty liver and insulin resistance can be associated with increased androgens, the responsiveness of the adult liver to androgens, in the absence of increased circulating testosterone concentrations (Chapter 6), was examined. AR protein was found to be expressed in the nuclei, and more weakly in the cytoplasm, of hepatocytes across the parenchymal region (Fig. 9.3.A), as well as in the nuclei of cells associated with portal veins (arrow; Fig. 9.3.A). Interestingly, while a strong nuclear stain was observed in many hepatocytes, a large proportion of cell nuclei were also negative for AR (arrow; Fig. 9.3.B). This pattern did not appear to be altered by prenatal androgens. QRT-PCR analysis revealed an up-regulation of *AR* in the livers of prenatally TP-exposed adult sheep ($P<0.05$; Fig. 9.3.C), suggesting an increased receptivity to this steroid. There was no difference in expression of genomic *ERα* (Fig. 9.3.D) but there was an increase in the hepatic expression of *GR* ($P<0.05$; Fig. 9.3.E).

9.3.4 Functional changes in the adult liver

To determine whether there were functional changes in the liver itself that may be associated with metabolic dysregulation the effect of prenatal androgens on hepatic gluconeogenesis was examined by assessing liver PEPCK expression. Immunolocalisation of PEPCK (Fig. 9.4.A) revealed widespread protein expression throughout parenchymal liver tissue with positive staining visualised within the cytoplasm and membrane of hepatocytes particularly in the peri-portal region. The liver capsule, comprising connective tissue, was negative for PEPCK (arrow; Fig. 9.4.A) as were the non-hepatocyte cells surrounding the hepatic portal vein (HPV and arrow; Fig. 9.4.B). However, there were no changes in adult hepatic *PEPCK* expression in maternally TP-treated animals (Fig. 9.4.C). There were transcriptional differences in the components of hepatic IGF system in prenatal TP-exposed adults, where *IGF1* was up-regulated ~3-fold ($P<0.01$; Fig. 9.4.D) without any change in *IGFBP1* expression (Fig. 9.4.E). There was a non-significant trend for hepatic IGF1 receptor (*IGF1R*) to be increased after maternal TP-exposure ($P=0.068$l; Fig 9.4.F).

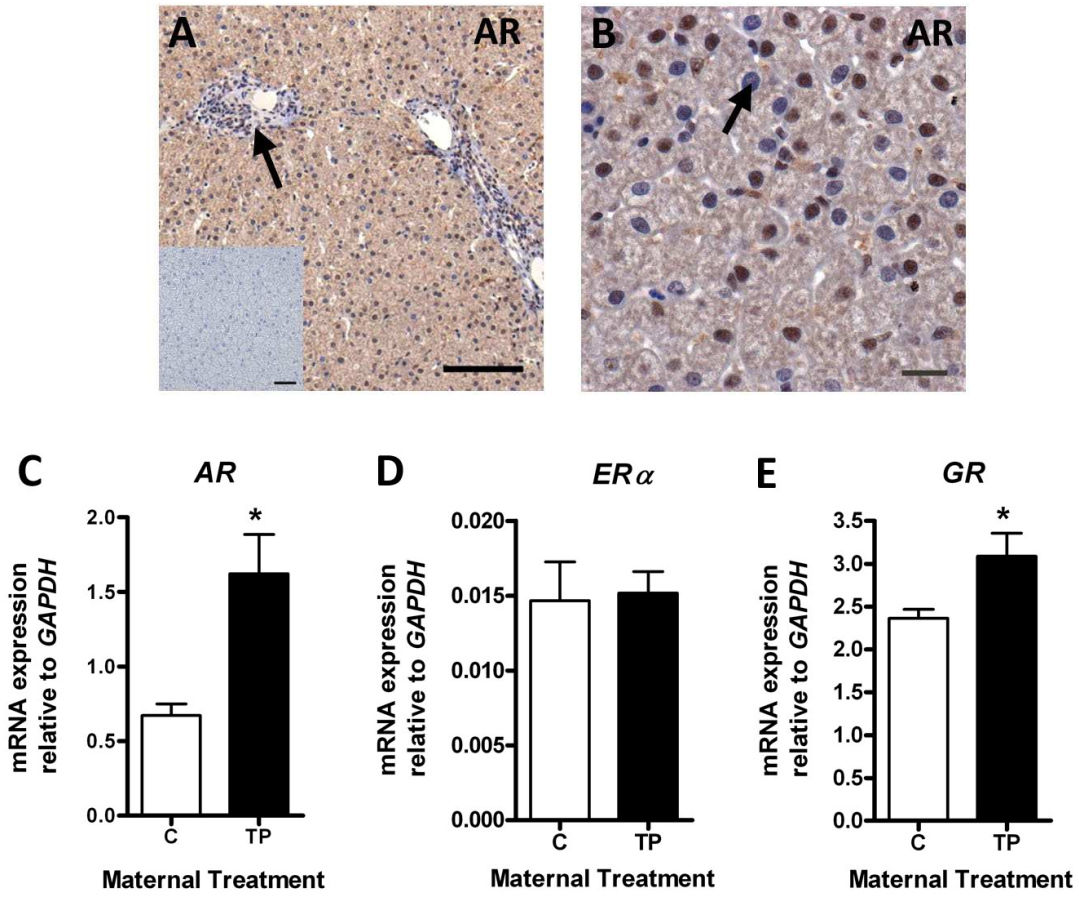

Figure 9.3 The effect of maternal prenatal androgenisation on adult liver steroid receptor expression. A) and B) Immunohistochemistry showing androgen receptor (AR) localisation in control adult ovine liver sections (A, scale bar = 100µm; arrow indicating staining in blood vessels and B, scale bar = 20µm; arrow indicating negatively stained hepatocyte nucleus). Inset in A) is negative control, scale bar = 50µm. QRT-PCR showing the effect of prenatal maternal androgenisation on hepatic *AR* (C), *ERα* (D) and *GR* (E) mRNA expression.* $P<0.05$.

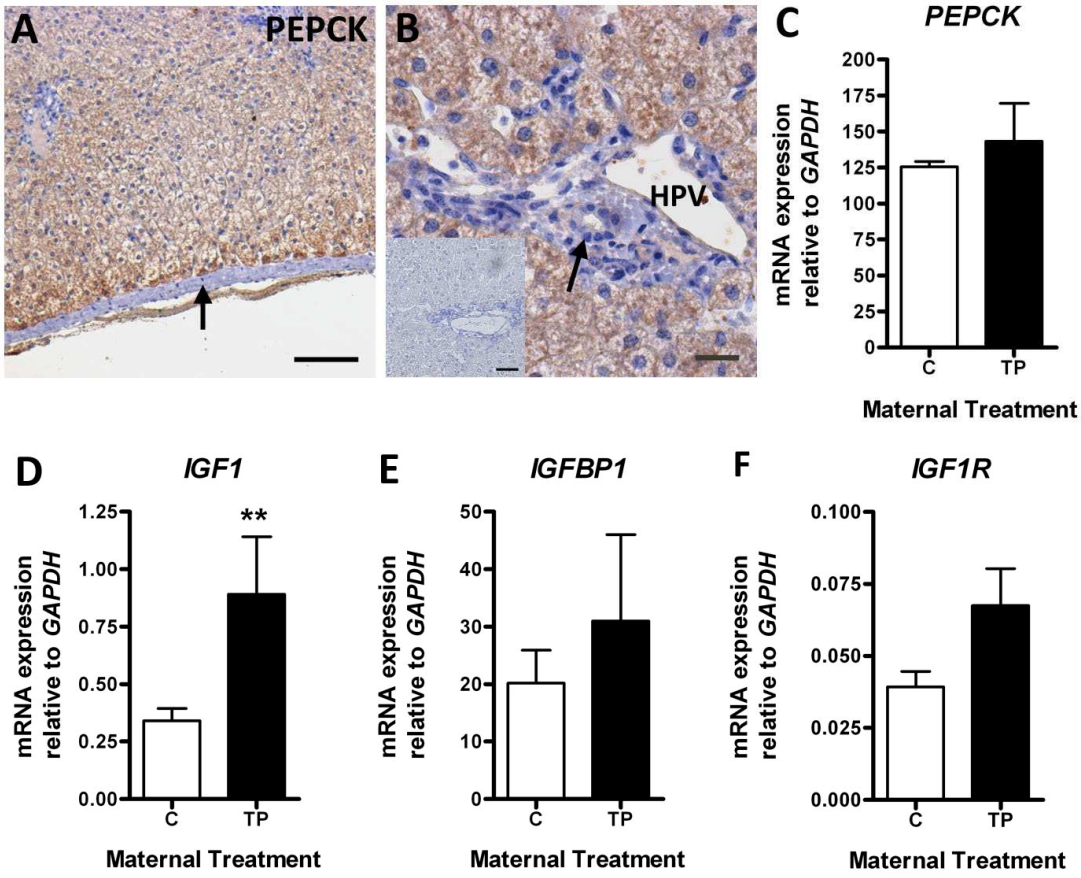

Figure 9.4 The effect of maternal prenatal androgenisation on hepatic metabolic and signalling pathways. A-B) Immunohistochemistry for PEPCK (brown) in control adult liver sections. Scale bars in A and B are 100µm and 20µm, respectively. Inset in B is a negative control, scale bar = 50µm. QRT-PCR showing the effect of prenatal maternal androgenisation on *PEPCK* (C), *IGF1* (D), *IGFBP1* (E) and *IGF1R* (F) mRNA expression. ***P*<0.01.

9.3.5 Fetal exposure to testosterone *per se* might not be responsible for the subclinical fatty liver change

To assess the effects of prenatal androgenisation without the contribution of placental metabolism of TP, the metabolic and liver outcomes were also investigated in adult offspring that were directly treated with TP *in utero*. Fetuses were directly exposed to TP or vehicle control by ultrasound-guided injection at d62 and d82 of gestation. There was no difference in the proportion of adult animals histologically categorised for fatty liver between TP treatment and control (Fig. 9.5.A). In fact, and unexpectedly, all animals showed signs of possible, or actual fatty liver (Fig. 9.5.A) and the prevalence of fatty liver was similar to maternal TP exposed animals rather than maternal control injection animals. Nevertheless, like the maternal treated cohorts, liver function determinants including serum AST (Fig. 9.5.B), GGT (Fig. 9.5.C), ALT and ALP (Appendix C) were not altered by direct fetal androgenisation. In addition there were no differences in central obesity (omental fat; Fig. 9.5.D), circulating leptin (Fig. 9.5.E), FFA, triglyceride (Appendix C) or cholesterol (Fig. 9.5.F) concentrations.

9.3.6 Insulin homeostasis in direct fetally treated adult offspring

There were no significant differences between direct fetal control and TP animals when glucose (Fig. 9.5.G) and insulin (Fig. 9.5.H) levels were determined by GTT in adult animals. This suggests that fetal TP exposure does not directly affect insulin homeostasis. However, like the changes in hepatic lipid content, the direct fetal intervention alone also affected insulin secretion in response to glucose load. Although baseline glucose was similar in the fetal and maternal treatment groups, 15 min after a glucose bolus the serum glucose concentrations in the fetal treatment group were approximately double those of the maternal treatment group (Fig. 9.5.G and Fig 9.2.H; Maternal control vs. Fetal control P=0.064). In addition, the fetal control insulin concentrations after 15 min were midway between the maternal control and TP treatments (Fig. 9.5.H and Fig. 9.2.I). Like the fatty liver findings, this implies that the fetal injection *per se* has a metabolic phenotype that is not changed by the presence of testosterone and may effect glucose and insulin regulation.

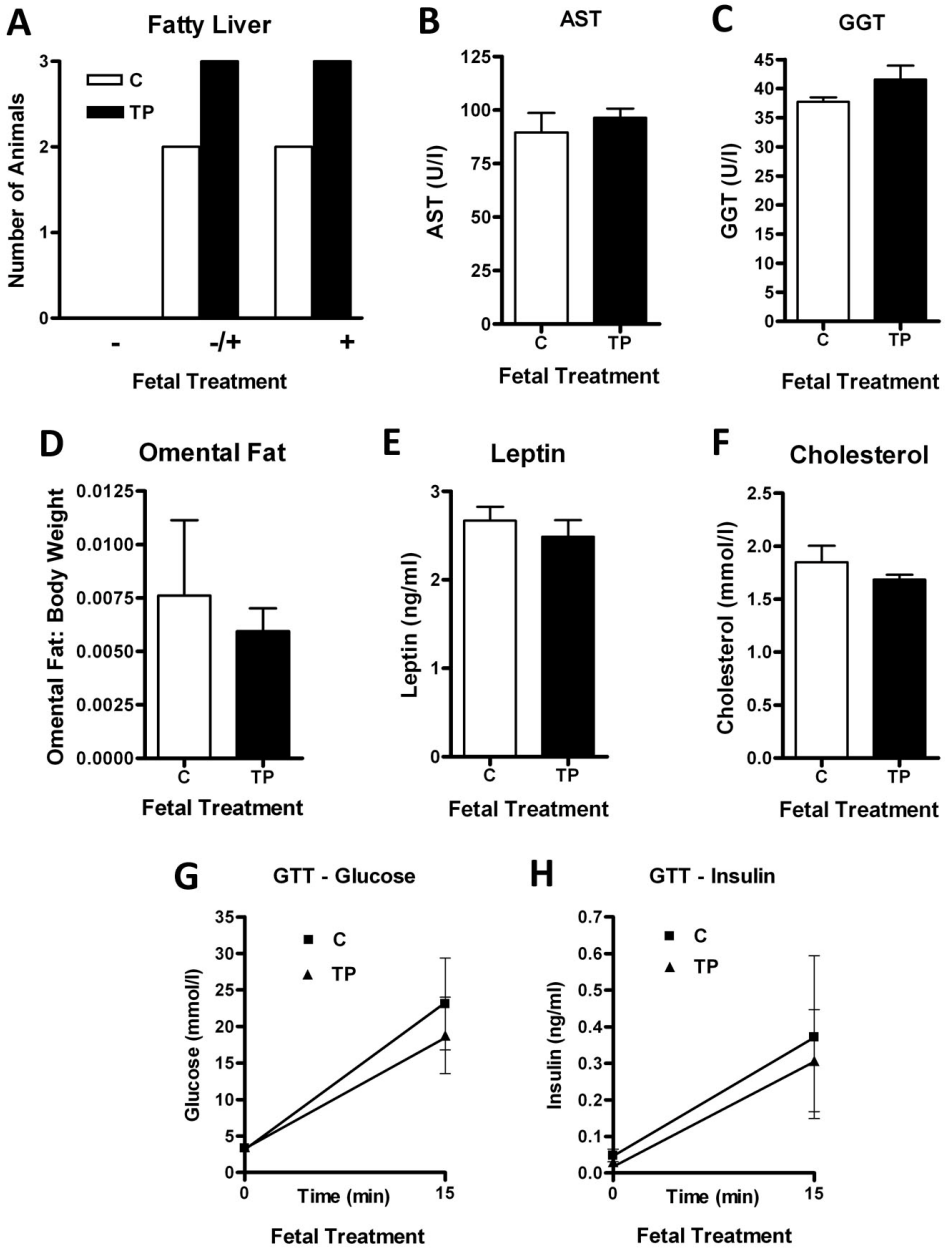

Figure 9.5 The effect of direct fetal androgenisation on metabolic parameters in adult offspring. A) Classification of fatty liver in directly androgenised C and TP cohorts. The effect of fetal prenatal TP treatment on adult liver analytes, including AST (B) and GGT (C), the weight of omental fat (D), plasma leptin concentrations (E), and cholesterol levels (F). Plasma glucose (G) and insulin (H) concentrations at basal levels and 15 min after i.v. glucose bolus.

9.3.7 Alterations in the expression of hepatic steroid receptors

Unlike the maternal TP-treatment cohort, hepatic *AR* gene expression was not increased (Fig. 9.6.A), in fact it showed a tendency to be reduced, although this did not reach statistical significance (P=0.085). In addition, in contrast to the maternal treatment, direct fetal-TP treatment led to the up-regulation of *ER*α gene expression in adult livers (P<0.05; Fig. 9.6.B). No change in hepatic *GR* mRNA expression was observed in these animals (Fig. 9.6.C).

9.3.8 Functional changes in the adult liver

Adult liver *PEPCK* mRNA expression was down-regulated following direct fetal TP exposure (P<0.05; Fig. 9.6.D), in contrast to findings in the androgenised maternal cohort. However the phenotypic effect of *in utero* injection was once more highlighted by a significant up-regulation of *PEPCK* mRNA levels in fetal control compared to maternal control (P<0.05). In contrast to maternal treatment, direct prenatal androgen exposure did not lead to significant alterations in the hepatic IGF system. There were no changes in *IGF1* (Fig. 9.6.E), *IGFBP1* (Fig. 9.6.F) or *IGF1R* (Appendix C) gene expression.

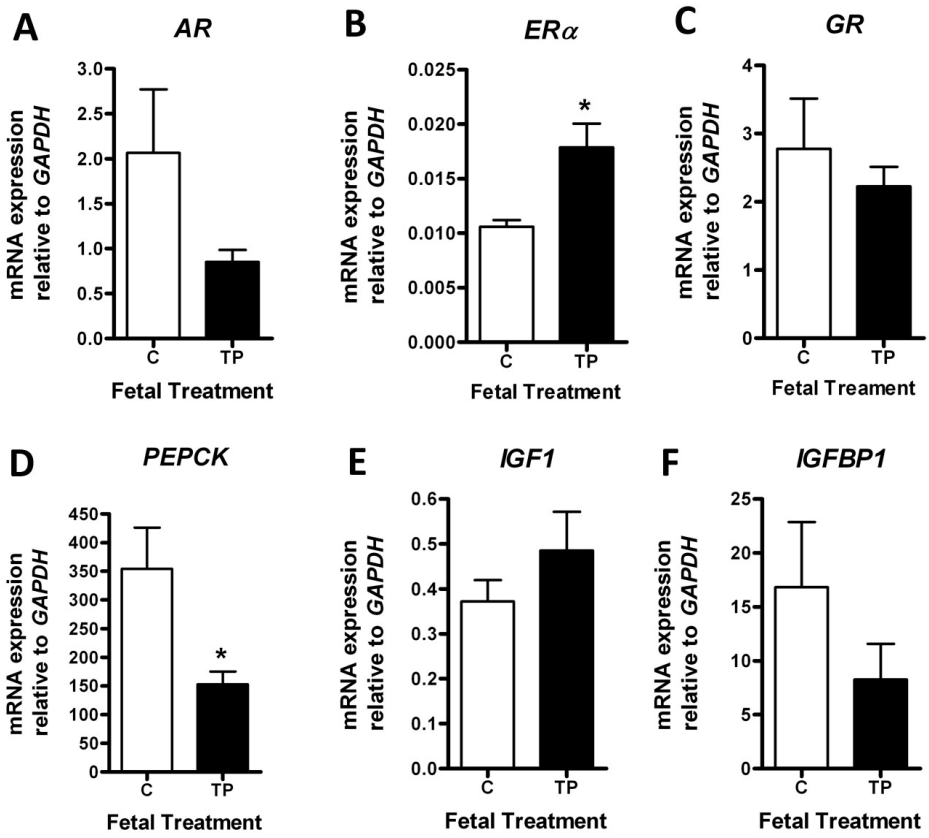

Figure 9.6 The effect of direct fetal androgenisation on molecular liver function in adult offspring. QRT-PCR data showing the effect of direct prenatal fetal androgenisation on hepatic *AR* (A), *ERα* (B), *GR* (C), *PEPCK* (D), *IGF1* (E) and *IGFBP1* (F) mRNA expression. **P*<0.05.

9.4 Discussion

This chapter focussed on the fetal programming effects of androgens on the adult liver, a key organ in regulating metabolic homeostasis, and also the physiological effect of prenatal treatments on insulin secretion and response to glucose load. Maternal prenatal androgenisation led to subclinical hepatic steatosis in young animals. Importantly this fatty liver was not a general reflection of central fat accumulation as it was not associated with an increase in omental fat. In addition, overall fat mass as assessed by body weight (Chapter 3) and plasma leptin concentrations were unchanged. Clinically, liver damage was not discernable as standard liver function tests remained normal. If these findings inform us about PCOS this suggests that liver abnormalities are evident at an early stage before any obesity or hyperandrogenism is manifest.

While fatty liver has not been previously reported in studies of prenatal androgenisation, NAFLD is associated with PCOS. Women with PCOS have increased prevalence of fatty liver that is associated with elevated aminotransferases, hyperandrogenaemia and central obesity (614-616, 618, 619). It is therefore plausible that early hepatic metabolic disturbance may in fact precede weight gain that would act to enhance liver damage and that subclinical fatty liver is a feature that worsens with obesity and hyperandrogenaemia. Interestingly, one study observed that a significant proportion of lean PCOS women also developed fatty liver (618) and another study failed to confirm significant central fat accumulation in obese women with PCOS (620). As these sheep showed evidence of pancreatic dysregulation it remains unclear whether there is a temporal relationship or association between the development of fatty liver and insulin resistance (621).

Although the development of fatty liver in young adults may be programmed in the fetus, the causative role of prenatal androgens is not clear. Direct fetal exposure to TP also led to hepatic lipid accumulation; however, control animals also presented physical signs of fatty liver, indicating a phenotype arising solely from the fetal treatment intervention that could have a stress, inflammatory or metabolic origin. It is clear however that high concentrations of fetal androgens (Chapter 3) did not programme additional fatty liver changes. This finding implies that the changes observed in the maternal treated cohort may not necessarily be attributed to the actions of androgens and confirms that not all of the features seen in

prenatal androgenisation animal models of PCOS are directly related to androgen exposure (335).

Insulin resistance and hyperinsulinaemia are common features of PCOS that are augmented by the associated obesity, but are also intrinsic without the requirement for increased central fat or BMI (167, 168). In this regard, maternal TP-exposed young adult sheep exhibited increased basal serum insulin levels that although were not significantly altered, may indicate early signs of pancreatic β-cell hypersecretion, known to be a cause of hyperinsulinaemia in PCOS (167, 622). Moreover, insulin response to glucose load was significantly increased in maternally androgenised animals. Subsequent glucose clearance was not affected in these animals suggesting that while early signs of insulin resistance may be present, these animals are not glucose intolerant. Although for technical reasons we were unable to conduct a euglycaemic clamp on these animals this finding further underlines intrinsic changes that occur before any alterations in body weight, fat distribution or circulating androgen concentrations (Chapter 6).

Previous studies have shown that prenatal TP from d30-90 and/or d60-90 lead to reduced insulin sensitivity in 5 week (478) and 11 week (479) old lambs and that the critical developmental programming period for insulin signalling is during d60-90 (479). Additionally, these studies reveal the parallel effects of prenatal TP and non-aromatisable DHT indicating an androgenic mode of programming (479). In this study however, basal insulin secretion as well as glucose clearance and insulin secretion following a GTT, were not affected by direct fetal TP treatment. The similarity of maternal TP and DHT effects in other studies, imply that fetal estrogen is not involved in the development of insulin resistance. However the lack of an effect by the direct fetal injection of TP, that massively increases fetal TP exposure (Chapter 3), questions the role of androgens. Of course it is possible that the changes that occur in response to the injection itself may mask or alter any further alterations induced by androgenic programming and therefore it is difficult to draw robust conclusions in this regard.

The finding of altered adult liver tissue composition prompted the investigation of its role in metabolism and glucose homeostasis. Hepatic cytosolic PEPCK catalyses the conversion of oxaloacetate into phosphoenolpyruvate and is the rate-limiting step of gluconeogenesis (421). Gluconeogenesis is an essential hepatic process that maintains normal blood glucose

concentrations. PEPCK undergoes tight transcriptional control so that glucose production is appropriately managed and can be regulated by hormones, including insulin that has an inhibitory role and glucagon and glucocorticoids that stimulate its expression (422). It is therefore a prudent candidate molecule for dysregulation associated with the metabolic phenotype of prenatal androgenisation.

Although fetal androgenisation through the maternal route was associated with insulin resistance, there was no effect on hepatic *PEPCK* expression in the adult ewe. However, direct *in utero* treatment led to differential changes in *PEPCK* gene expression with a significant up-regulation in fetal control compared to maternal control animals, but a down-regulation in fetal TP compared to fetal control cohorts. In this instance the fetal injection phenotype was altered by direct exposure to androgens. In mice, the over-expression of *PEPCK* leads to a type II diabetic phenotype (623), which would be expected given the role of PEPCK in regulating glucose synthesis. However liver-specific knock-out of this gene did not, unexpectedly, affect blood glucose and remarkably did result in the development of fatty liver (624), suggesting a role in liver lipid metabolism distinct from gluconeogenesis. This has subsequently been explained by the hepatic role of PEPCK in cataplerosis, a metabolic pathway involving the removal of intermediary unoxidisable citric acid cycle anions from the Krebs cycle (625). Mice with a liver-specific deletion of cytosolic *PEPCK* developed fatty livers on postnatal day 1, thought to be due to the reduced ability of the liver to oxidize fatty acids resulting in an increase in triglyceride synthesis and hepatic fat accumulation (626). The role and regulation of liver *PEPCK* after fetal programming is complex and requires further work but it can be altered by fetal injection and modified by androgen exposure in the fetus.

The effect of prenatal androgenisation on liver pathways involved in growth, development and metabolism were also examined. The expression of hepatic *IGF1*, *IGFBP1* and *IGFR1* was studied in young adults. IGFBP1 sequesters IGF1 reducing bioavailability, thus controlling ligand-receptor binding potential (627). An increased IGF1:IGFBP1 ratio (628) and reduced IGFBP1 (629) in serum is associated with obesity, hyperinsulinaemia and insulin resistance in human studies. Reduced circulating IGFBP1 has also been linked to PCOS in women, although this is likely attributed to the metabolic, rather than reproductive, aspects of the syndrome (630, 631). In this study, hepatic *IGF1* mRNA was significantly increased in indirectly androgenised offspring suggesting that alterations in the hepatic

expression of IGF1 may be involved in the phenotype associated with ovine prenatal androgenisation (380, 478, 479).

There was no alteration in adult hepatic *IGF1* gene expression after direct fetal injection of TP. This implies that androgens might not be involved directly in this programming and highlights that placental androgen metabolites, such as estrogen, may be involved. It is likely that steroids have a role in the modulation of liver IGF1 expression. The human *IGF1* gene is known to contain androgen responsive elements in the upstream promoter region that bind AR and stimulate *IGF1* transcription (632). Although circulating testosterone is not altered in these sheep, liver *AR* is increased after prenatal androgen exposure by maternal TP administration, suggesting that this organ is more responsive to androgens. Speculatively, the increase in *IGF1* expression and up-regulation of *AR* in the maternal treated ovine liver may be associated. Unfortunately because of the recognised technical limitations of Western blotting for IGF1 or AR in ovine tissues it was not possible to convincingly and robustly analyse expression at the protein level.

As alluded to, the up-regulation of liver *AR* in adult sheep seems to originate from the estrogenic fetal programming effects of TP since this effect does not occur after direct fetal TP exposure. Conversely, analysis of liver *ERα* gene expression revealed an up-regulation in directly, but not indirectly TP-exposed adults. Consequently, it is testosterone that may programme the expression of the liver estrogen receptor. It is not known what the significance of these changes in hepatic receptivity to these steroid hormones is, but illustrates intrinsic perturbations in liver function and signalling as a consequence of prenatal androgenisation.

Exposure of an adult to increased glucocorticoid concentrations is associated with insulin resistance, hyperglycaemia and central obesity (633). Interestingly hepatic *GR* shows a small but significant up-regulation after maternal androgenisation of the fetus. This does not occur following direct exposure to androgens. Overall this implies that fetal steroids can permanently programme liver steroid reception. If this were the case our data suggest that the effects on hepatic *AR* and *GR* may be mediated by estrogen while androgens have a direct effect on the adult liver expression of *ER*, although these results must be treated with caution given the underlying liver phenotype present in the direct fetally treated animals.

For the first time, the occurrence of hepatic steatosis and early indications of liver damage in an ovine model of prenatal androgens is reported. These changes are independent of central obesity and may be the culmination of disturbances to hepatic metabolic homeostasis predisposed by exposure to *in utero* factors, including androgens, estrogens and/or physical stress. In addition indirect maternal TP exposure results in increased hepatic receptivity to androgens, in the absence of hyperandrogenaemia, and is also associated with the up-regulation of liver *GR* and *IGF1* mRNA expression as well as early indications of insulin resistance. Direct prenatal androgenisation leads to increased receptivity to estrogens. In addition, the changes observed in liver *PEPCK* transcription following direct prenatal vehicle or TP administration, may indicate altered liver glucose pathways that could be associated with the occurrence of fatty liver that arise through impairment of alternate hepatic metabolic pathways. These animals also appear to have metabolic disturbances relating to glucose clearance and insulin response.

It is clear that the pathways involved in programming metabolic dysregulation in the adult are complex and not easily dissected, nevertheless the early modifications to liver function present in these young adults may precede weight gain and hyperandrogenaemia that are common in women with PCOS. It seems that the hormonal or peripheral phenotype of the maternally androgenised ewe may not, in fact, be entirely normal in these young adults. It is therefore possible that the enhanced environmental factors such as insulin or IGF signalling may be linked to the effects of augmented androgen secretion by the ovary and the adrenal gland, however this is not known. Furthermore, it is clear that there is a fetal injection phenotype that mirrors some of the effects of maternal androgenisation, in the absence of androgens. The findings from these studies, how they add to the literature, and how they can be taken forward are discussed in the following chapter.

Chapter 10

General Discussion

The clinical features of PCOS have been recognised in women for many years (161), and have become more defined over recent times as medicine searches for the answers to the aetiology and the appropriate lines of therapy (163). Ovulatory dysfunction is common in the modern woman and this is confounded by environmental factors such as lifestyle. The high prevalence of obesity in women with PCOS only acts to worsen the overall milieu of hormonal, ovarian and metabolic disturbances (163). In addition, the high levels of circulating androgens, which promote the accumulation of fat in a male-like central distribution, also have superficial manifestations including severe acne and hirsutism. These features, in combination with reproductive elements, are particularly distressing and are known to lead to lowered self-esteem, anxiety and depression in some women (634, 635) and therefore have a major social impact on their quality of life.

Medical and scientific observations have unravelled many of the pathways that are associated with PCOS demonstrating the multifactorial and complex nature of the disorder, yet the underlying cause(s) remain unknown. Within the last twenty years standard guidelines have been set down to provide clinicians with a framework to diagnose PCOS (173, 174), however, even with these in hand it is clear that PCOS is not a clear-cut disorder and there are many diversities of this syndrome in women. While there have been numerous candidate genes for linkage to PCOS (225), none have been robustly and reproducibly correlated to all of the main features of PCOS and genetic influences remain unsolved. The most promising research into PCOS has involved the modelling of a PCOS-like phenotype in non-human primates, rodents and sheep that indicate an early programmable developmental origin of this disorder (304). Such models are established through the fetal programming effects of steroid hormones that are likely to induce epigenetic modifications to the genome.

10.1 The ovine model of mid-gestation prenatal androgenisation

The exposure of an ovine fetus to increased concentrations of androgens during development leads to multiple reproductive, neuroendocrine and metabolic aberrancies that are reminiscent of PCOS in women. The timing of prenatal exposure does however appear to be key to the development and severity of some of these traits. Those female animals that are exposed from early to mid-gestation (d30-90), spanning the period of sexual differentiation of the reproductive tract and brain, develop the most severe reproductive and endocrine effects (352, 372-374, 378, 379). Furthermore, female offspring are born with ambiguous

genitalia, possessing empty scrotal sacs and a penis yet retaining ovarian development (370, 373, 495). In the later model of prenatal androgenisation, which spans mid-gestation in the sheep (d60-90), the reproductive/endocrine effects appear to be less severe (299); however, certain metabolic effects of prenatal androgens are still prominent (479). The crucial difference between this early and late model of prenatal androgenisation in the sheep is the absence of external masculinisation in the later exposed female, born without male-features and possessing normal AGDs (Chapter 3) (370). The lack of virilisation in this model makes it more relevant to the normal anatomical arrangement of the genital tract present in women with PCOS.

The major reprogramming of the brain that is present in the ovine d30-90 androgenisation model makes it difficult to dissect out subtle changes that contribute to an overall phenotype, with those of the prominent masculinisation effects that perturb adult reproductive function in the female. In these animals there is severe hormonal imbalance and endocrine function is disturbed (352, 379, 380), and while these are features of PCOS they have not necessarily arisen in the same manner. The assessment of mid-gestation prenatal androgen exposure, from d62-102, in this thesis, revealed that in young adult female offspring, circulating sex steroids and gonadotrophin dynamics were normal, ovarian cyclicity was not altered between control and treatment groups and PCO was not present in TP-exposed ewes during the first breeding season (Fig. 10.1). Abnormal vaginal development, as well as a male urinating posture, as biomarkers of testosterone/estrogen activity during development, does however clearly demonstrate the occurrence of prenatal exposure to sex steroids in these offspring.

The mid-gestation exposure of an ovine fetus to increased androgens generates a model that does not fulfil the major diagnostic criteria for PCOS in young animals, and therefore the animals studied here do not yet have experimentally-induced PCOS. This provides a unique opportunity to study the effects of prenatal androgens without the influence of confounding factors such as hyperandrogenaemia, LH hypersecretion and polyfollicular ovaries, with the possible exception of slightly raised peripheral insulin concentrations. A thorough assessment of ovarian follicular, adrenal and liver function as well as glucose/insulin homeostasis revealed that changes are apparent as a consequence of prenatal androgenisation (Fig. 10.1) that may represent primary, rather than secondary, defects of a PCOS-like syndrome.

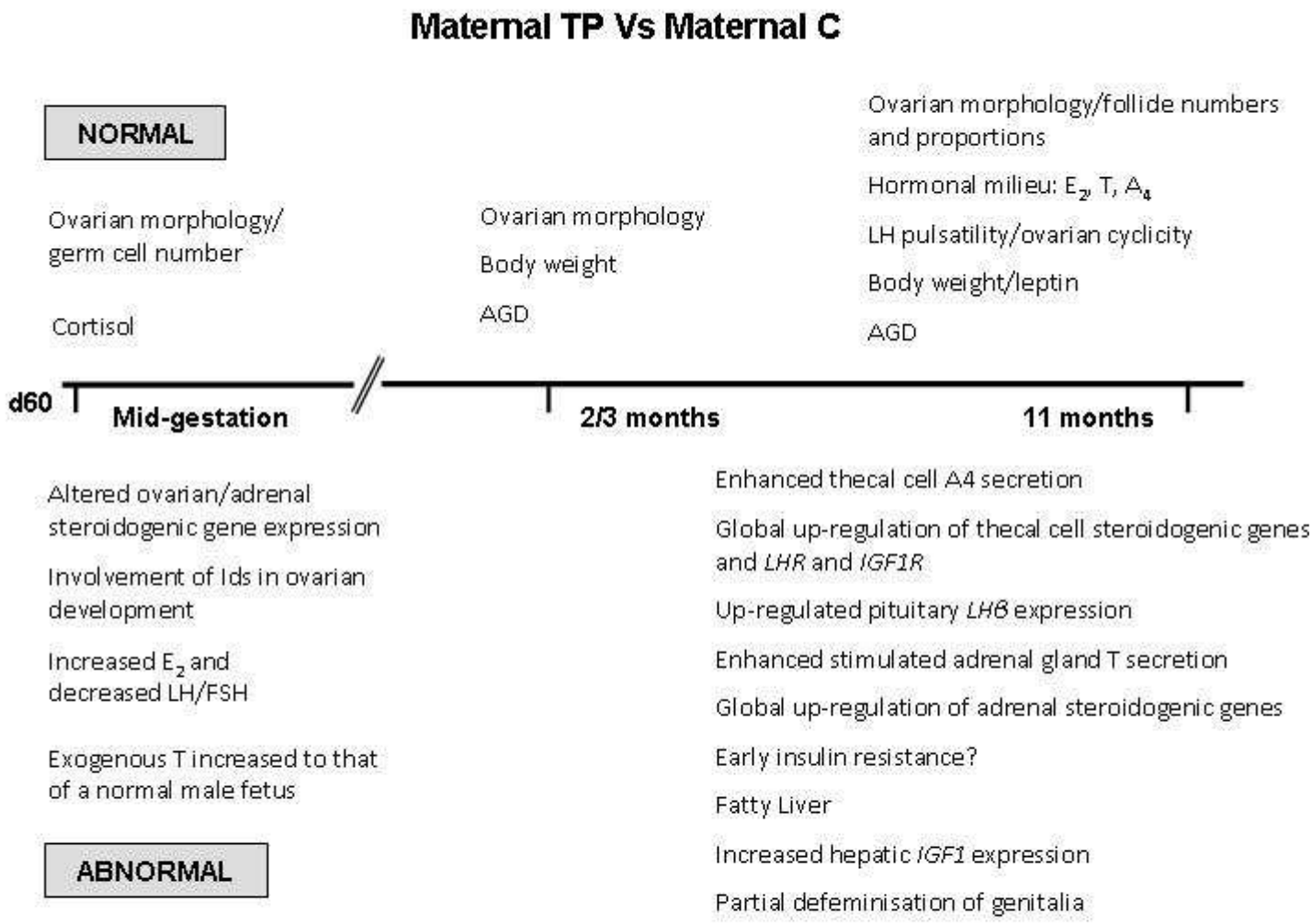

Figure 10.1 Summary of the main findings in animals that were indirectly maternally androgenised *in utero*. The effects of mid-gestation maternal TP treatment are compared with control animals. Exposure to TP resulted in certain fetal and adult physiological aspects which were normal and others that were abnormal. C: control, E_2: estradiol, T: testosterone, A_4: androstenedione.

In PCOS, hyperandrogenaemia arises through excessive androgen production by both the ovary (344) and the adrenal gland (414). In the mid-gestation ovine model of prenatal androgenisation, it has been demonstrated for the first time that there is programming of a follicular phenotype that is independent of androgen and gonadotrophin signalling in the adult. The thecal cells from such animals have an unequivocal pre-disposition to secrete increased concentrations of the androgen, androstenedione, and do so with and without exogenous stimulation with LH. This drive for enhanced steroidogenesis is identified at a molecular level in which there is a global up-regulation of genes that are crucial components of the steroidogenic pathway, including *StAR*, *CYP11A*, *HSD3B1* and *CYP17*. In addition, there is up-regulation of thecal cell *LHR*, which renders the thecal cell more responsive to the steroidogenic effects of LH and a concomitant increase in pituitary *LHB* subunit expression, although this does not reflect changes in peripheral LH concentrations at this stage. These novel data from an animal study of PCOS are in line with observations in thecal cells that are collected from the ovaries of women with PCOS, in which the findings are remarkably similar. The women in these studies are however hyperandrogenic, have distorted gonadotrophin profiles and PCO, making it difficult to determine the primary effects of PCOS on the ovary, from the secondary effects of hormonal imbalance. The studies outlined in this thesis provide evidence for an intrinsic programming event in ovarian cells, which could include the pre-thecal cells that are present in the developing ovary.

In fact, study of the ovine fetal ovary after ~10 and ~30 days of androgen exposure does indicate direct programming effects on this tissue during this time. Perturbations to the steroidogenic biosynthesis capacity with a general down-regulation of many of the genes involved in this pathway were observed. It is not known in which cell type(s) these changes occur, how this might affect the fetal ovary and whether the local production of steroid hormones is negatively affected by the large peripheral levels of exogenous testosterone and a subsequent increase in estradiol, during this time. The alterations in gene expression may be important and lead to altered autocrine or paracrine signalling between somatic cells that programme permanent functional changes. In addition, it was demonstrated for the first time that the Ids are expressed, and may have important functional roles, in the fetal ovine ovary. Furthermore, differential expression of the Ids in the TP-exposed ovary compared to controls was observed, with the up-regulation of Id1 protein in the androgenised cohort. This may indicate the presence of complex pathways that could also contribute to the setting down of an adult ovarian phenotype. It is also possible that there is some involvement of the

gonadotrophins in the development of the female gonad as both *LHR* and *FSHR* are expressed in the mid-gestation ovary and it was found that pituitary secretion of both LH and FSH were dampened in these fetuses.

In the adrenal gland of prenatally androgenised adults, there was enhanced production of testosterone, after stimulation with ACTH, implying that the adrenal gland is also predisposed to excessively secrete androgens under certain circumstances. In women with PCOS, hyperandrogenism occurs in part through enhanced stimulation of adrenal androgen secretion, including DHEA/S and androstenedione (408, 414) and this has also been demonstrated in the Rhesus monkey prenatal androgenisation model (415). Secretion of one adrenal androgen, androstenedione, was not altered in our sheep model, which might explain why there was no detectable alteration in peripheral levels of this hormone in the young adult ewe. Cortisol secretion was also unaffected by prenatal treatment, implying shunting of hormone biosynthesis towards the testosterone pathway. Indeed, molecular analysis of the adult adrenal gland revealed that steroidogenic gene expression was up-regulated, resembling the findings in the ovarian thecal cell. These changes were also independent of alterations in pituitary *POMC* expression, implying that these are also intrinsically programmed changes that are not influenced by other factors, such as endogenous ACTH. The ACTH receptor was not measured in the adrenal however, and would be useful to study to determine whether increased stimulation by ACTH could occur at the adrenal level. In the fetus, prenatal androgenisation for ~30 days resulted in the up-regulation of both *CYP11A* and *HSD3B1* in the d90 adrenal and therefore the adult phenotype may be linked to programming effects of androgens or estrogens during development.

In addition to the intrinsic effects of mid-gestation androgenisation on the endocrine glands of adults, there were also metabolic features present in these young animals. Basal insulin concentrations were slightly, but not-significantly raised in the TP-exposed cohort and when these animals received bolus glucose, insulin levels were significantly enhanced compared to controls. At the same time, glucose clearance was unaffected, implying that while there may be early signs of a hyperinsulinaemic response to glucose, peripheral glucose uptake is normal. It is therefore likely that these animals are exhibiting early signs of pancreatic β-cell dysfunction, an area that is ripe for future study. Abnormalities of pancreatic insulin secretion are proposed to occur in women with PCOS, leading to hyperinsulinaemia, independent of obesity (167, 168). Augmentation of this phenotype occurs through weight

gain and can culminate in insulin resistance (168, 452). In our sheep model, it is likely that this phenotype will develop further as the animal matures, as the ewes in this study were very young and metabolic dysfunction is not a marked feature at this stage of ongoing development. These sheep were reared on a normal caloric diet and therefore were not likely to gain weight inappropriately. Indeed, over-feeding sheep that have been prenatally androgenised from d30-90 considerably exacerbates the reproductive (482) as well as the metabolic aspects (479) of the PCOS-like phenotype, when compared to over-fed and normal weight controls. This is therefore another example of changes that may be inherently present but surface fully when hypercaloric environmental conditions are present.

In the d62-102 prenatal androgenisation model, sheep were not fat and they were not at this stage glucose intolerant. Nevertheless, examination of other metabolic tissues, such as the liver, did reveal stark physiological perturbations that could affect tissue function. There were early signs of fatty liver in the livers of TP-exposed adult ewes, an observation that has not yet been made in other animal models of prenatal androgenisation. This is relevant in that NAFLD is associated with insulin resistance and hyperandrogenaemia (613, 614) and is prevalent in women with PCOS (611, 614-616). Furthermore, the hepatic expression of *IGF1* was increased, which could impact on ovarian steroidogenesis (434), particularly as thecal cell *IGF1R* was concomitantly up-regulated. The fact that this phenotype was present without the accumulation of central fat or hyperandrogenaemia further demonstrates an independent modification of a physiological system that may reflect early overall aspects of a multifactorial disorder.

A hypothetical model of how mid-gestation prenatal androgenisation might affect different physiological systems in the adult ewe, with the ovary at the centre, is presented in Figure 10.2. It illustrates the establishment of a pre-PCOS model, in which there are a multitude of inherent tissue-specific changes that are present in the young adult ewe. In the ovary and adrenal at least, there are functional changes during development that occur through the direct actions of testosterone and/or estrogen. The propensity for androgen secretion by both the ovary and adrenal gland are increased and imply that the cellular machinery to promote this is in place before clinical manifestations are present. In addition, possible enhancement of insulin and/or IGF1 signalling may also directly act on the ovary, in an additive way to either affect ovarian steroidogenesis and/or follicular growth. Furthermore, there may be an effect of prenatal androgens on pituitary synthesis of LH that in the circumstances of

perturbed steroid or GnRH signalling, could contribute to the LH hyper-secretion or increased pulse amplitude that is observed in women with PCOS (164, 165) and animal models of PCOS (351, 352). It is possible that assessing the animals at this particular age (11 months) has allowed for the capture of these early changes that represent underlying mechanisms that are involved in the aetiology of PCOS-like characteristics that will develop further in this subtle mid-gestation model of prenatal androgenisation. For example, if the ovarian and adrenal defect were to develop further and result in increased peripheral concentrations of androgens, then it is likely that a hyperandrogenic state would act to augment the current phenotype present in the young adult ewe.

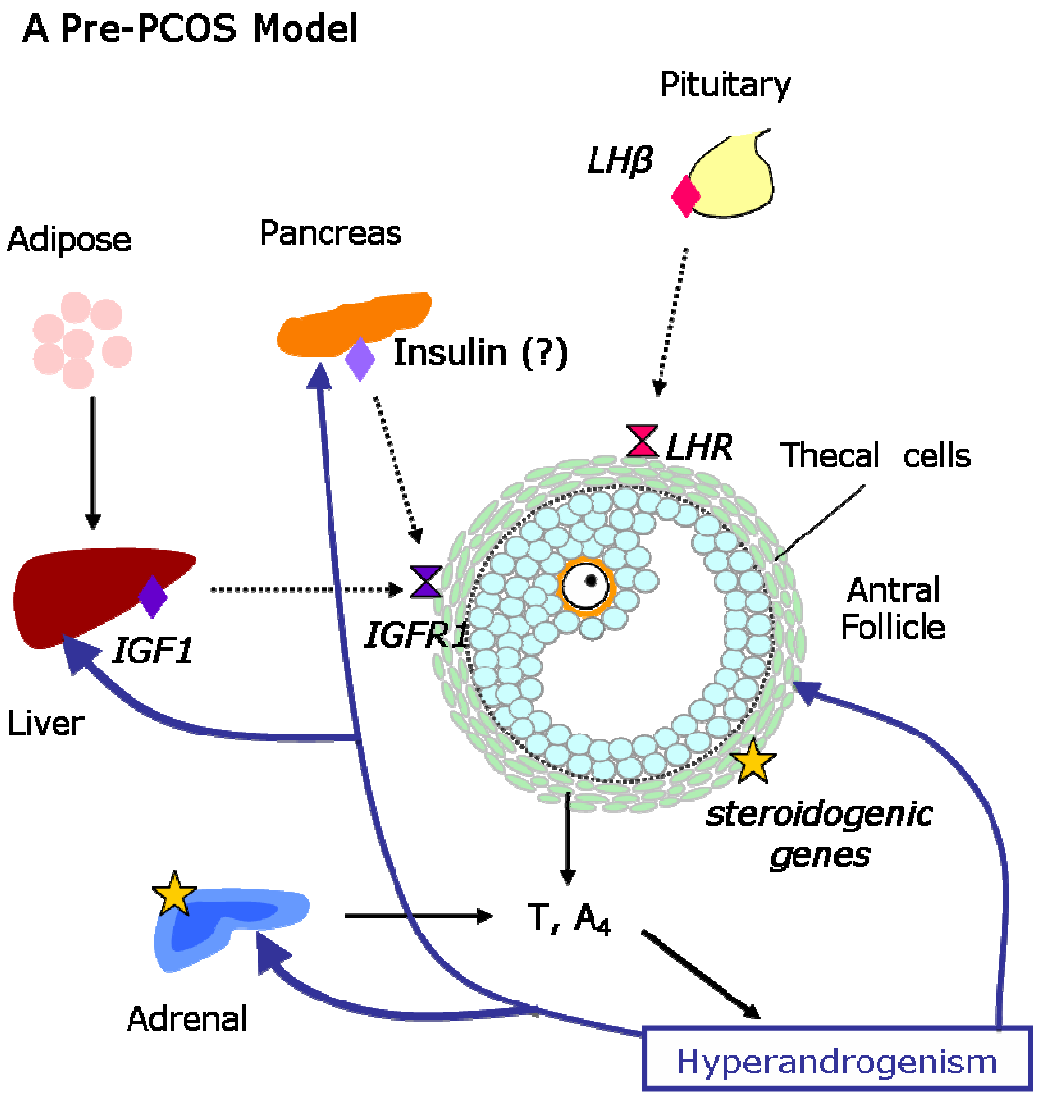

Figure 10.2 A pre-PCOS model is present in young adult sheep following mid-gestation prenatal androgenisation. Tissue-specific intrinsic gene expression and hormonal changes, and hepatic fat accumulation, are present in the maternally androgenised adult ewe, independently of severe ovarian, metabolic and endocrine features of PCOS. The dotted lines represent possible enhancement of signalling pathways. In the presence hyperandrogenaemia, increased androgens could negatively impact on the overall phenotype (blue arrows). A_4: androstenedione, T: testosterone.

10.2 The direct model of prenatal androgenisation

The direct delivery of TP to the ovine fetus was undertaken for 3 key reasons: 1) to bypass placental conversion of testosterone into estrogen metabolites, 2) to avoid any confounding effects of androgens on the mother that might indirectly affect the fetus, and 3) to control the dose of androgens to which each fetus is exposed. Hormonal analysis of fetal blood at the time of sacrifice confirmed that there were no increases in peripheral estradiol concentrations in TP treated fetuses, as were reported later in the maternally TP exposed cohort. A major inference from these studies was the remarkable resistance that the developing female fetus showed to frankly supraphysiological testosterone concentrations. Like the maternal cohort, these animals were exposed to androgens after the timing of sexual differentiation and masculinisation that would occur in male fetuses. Very few of these female animals exhibited abnormal vaginal development in adulthood, implying that such programming effects are mediated by estrogens. Conversely, all TP-exposed animals from fetal and maternal treated cohorts, exhibited male-like urination postures, a clear androgenic programming effect of which the critical period occurs during mid-gestation.

Like the maternally exposed cohort, directly androgenised adult ewes had not developed the definable features of PCOS that are described in women or animal studies, by 11 months of age (Fig. 10.3). The hormonal profile, ovarian cyclicity, LH secretion and pulsatility and ovarian morphology were not changed in comparison to controls or to the findings in the maternally exposed group. In parallel to the maternal cohort, direct prenatal androgenisation did exert some effect on ovarian androstenedione secretion and thecal cell steroidogenic gene expression. There were also direct effects of androgens on the developing ovary and adrenal gland. These changes could therefore be attributed to programming by either testosterone and/or estrogen. Unlike the maternal cohort, the fetal TP-exposed animals had possible dysregulation of hepatic gluconeogenesis due to down-regulated *PEPCK* expression. This differential finding might imply an androgenic mode of programming in the liver. Conversely, the up-regulation of *IGF1* gene expression, exclusively in the livers of the maternal TP-exposed adult ewe, suggests direct estrogenic programming. However, accurate delineation of the differential effects of androgens and estrogens in this model is confounded by the presence of a third steroidal hormone that may be raised during these fetal treatments, namely the stress hormone cortisol.

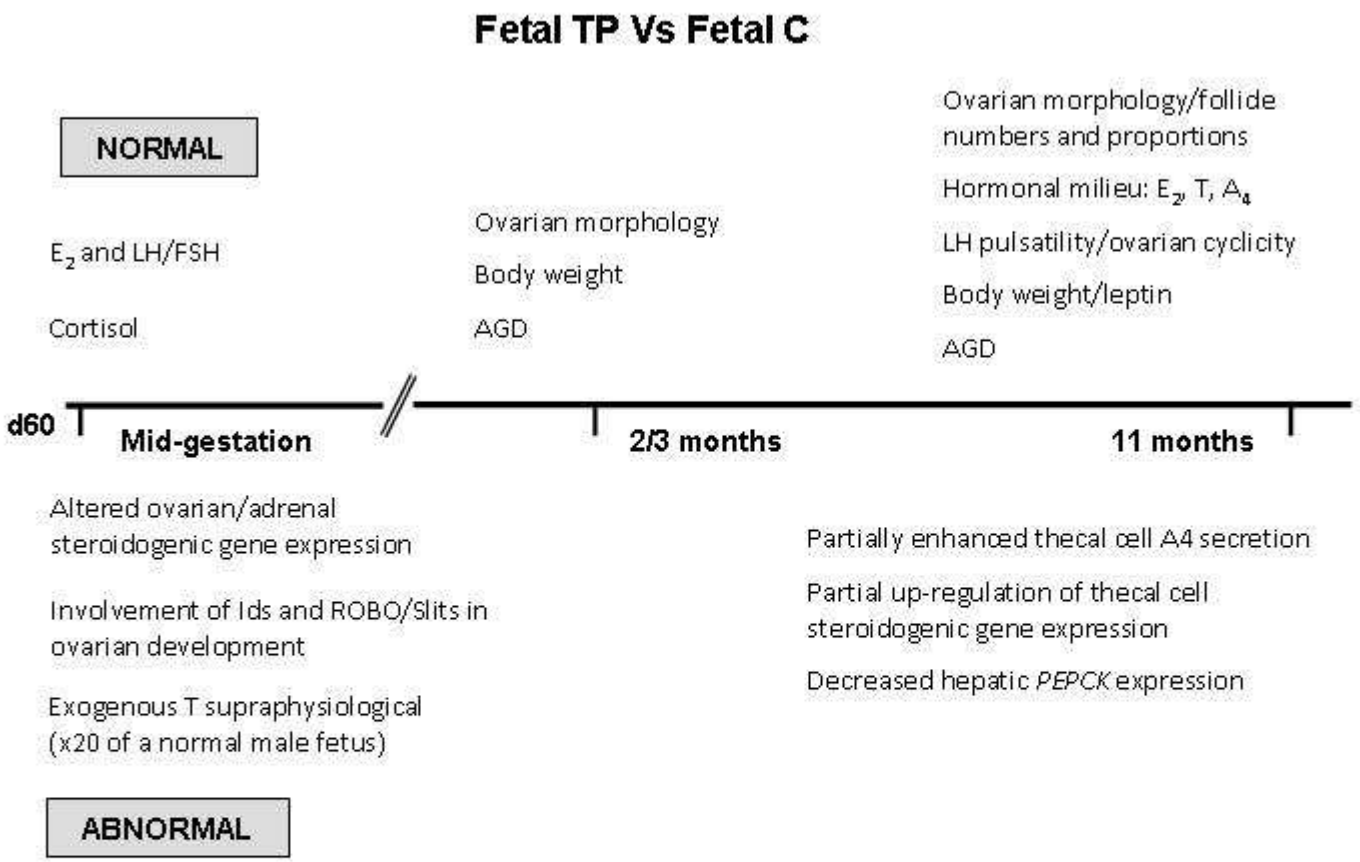

Figure 10.3 Summary of the main findings in animals that were directly fetally androgenised *in utero*. The effects of mid-gestation TP delivered directly to the fetus are compared with fetal vehicle control injections. Exposure to TP resulted in certain fetal and adult physiological aspects which were normal and others that were abnormal. C: control, E_2: estradiol, T: testosterone, A_4: androstenedione.

10.3 A non-androgenised model of PCOS

The comparison of circulating fetal cortisol levels at d70, between control and treated cohorts in the fetal injection group, did not reveal any differences (Fig. 10.3). However, comparison of the fetal injection control cohort with the maternal treated control group revealed that there was a trend for increased cortisol in the fetally treated groups (Fig. 10.4). As this measurement was carried out 10 days post treatment it is possible that initial activation of either the fetal and/or maternal HPA axis at this time, responsible for elevated peripheral cortisol concentrations, is dampened by d70. Nonetheless, this finding highlights an additional variable that is present in the fetal injection cohort and could confound the conclusions reached concerning androgenic and estrogenic programming effects.

Numerous features of the mid-gestation ovine maternal androgenisation model were also observed in the fetal injection control and TP cohorts, and were significantly altered compared to the maternal control animals (Fig. 10.4). One of the most considerable effects of the fetal injection paradigm was the excessive biosynthesis of androstenedione by the ovarian thecal cell, in which secretion was enhanced compared to both maternal control and TP animals *in vivo* and *in vitro*. In addition, the thecal cell expression of *StAR* and *CYP17* was up-regulated in this cohort pointing to perturbed intrinsic steroidogenic activity that, like the maternally androgenised cohort, is not influenced by alterations in the peripheral hormonal environment. The fetal injection model of steroid delivery also resulted in the development of a metabolic phenotype. All of the control (and TP) animals in the fetal injection cohort presented signs of possible or positive fatty liver. Furthermore, while direct fetal testosterone did not programme enhanced glucose-stimulated insulin secretion, comparison of fetal control and TP cohorts with maternal control and TP cohorts, revealed that in fetally injected cohorts, insulin secretion post glucose was in fact mid-way between maternal treatments.

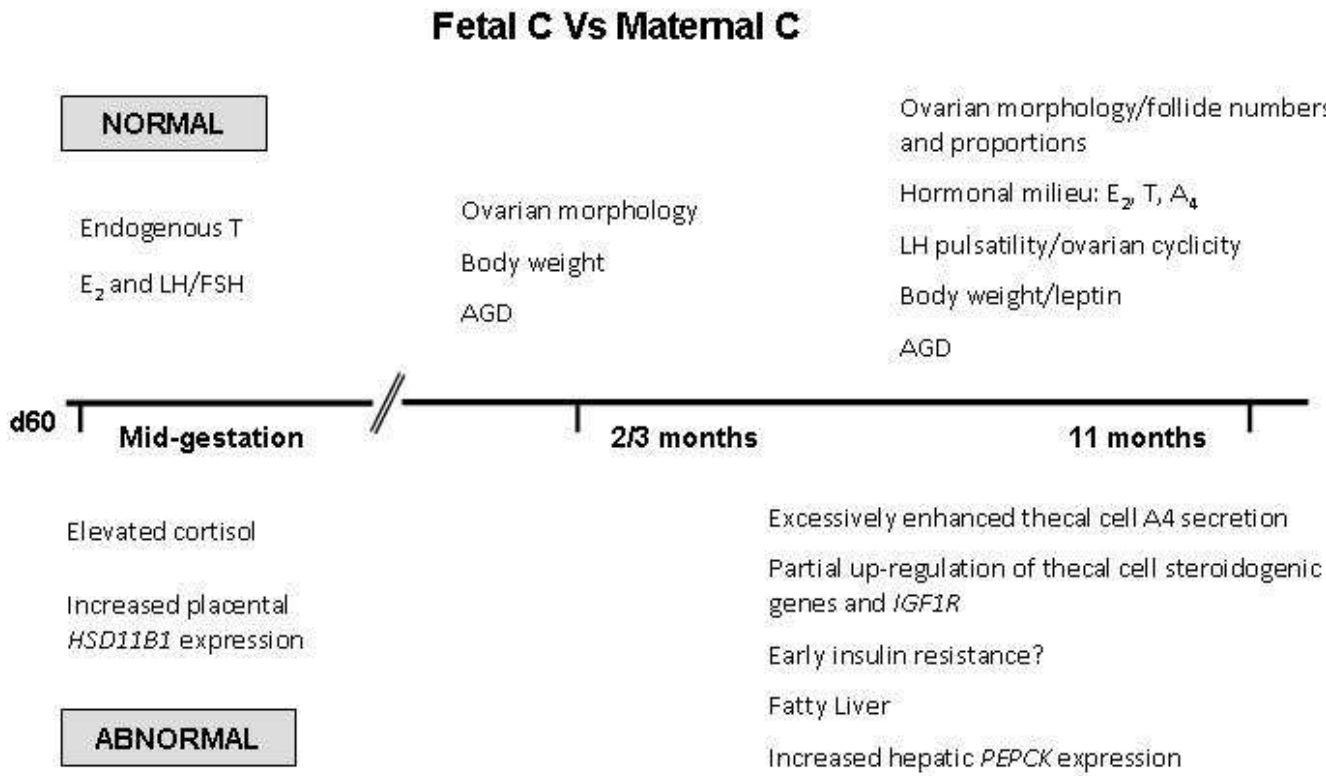

Figure 10.4 Summary of the phenotypic aspects of the fetal injection model in control animals. The effects of mid-gestation TP delivered directly to the fetus are compared with fetal vehicle control injections. Exposure to TP resulted in certain fetal and adult physiological aspects which were normal and others that were abnormal.
C: control, E_2: estradiol,
T: testosterone,
A_4: androstenedione.

The fact that these changes are generally present in both fetal control and TP cohorts presents a major caveat to this method of delineation of androgenic from estrogenic actions. It is not possible to robustly conclude whether it is the effects of glucocorticoids or androgens during mid-gestation might be responsible for these programming outcomes. In some cases it appears that the effects of fetal TP are additive to changes in the fetal control cohort, which might include *in vivo* thecal cell androstenedione production, suggesting that different pathways could programme an analogous adult ovarian phenotype. Conversely, differential hepatic gene expression of the gluconeogenic enzyme, *PEPCK*, in the fetal control and TP cohorts could point to separate programming effects of steroid hormones on liver glucose metabolism.

10.3.1 Potential sources of elevated fetal cortisol

Given the known role of glucocorticoid programming of fetal growth (286) and the development of obesity and related metabolic disorders in adult life (277, 278), cortisol is a promising candidate for the programming of some of the metabolic, and also ovarian, traits that are observed in the mid-gestation direct fetal injection cohort. Further evidence to support this is the elevated concentration of cortisol present in fetal blood ten days post-injection. It is possible that the physical injection to the fetal flank triggers a stress response and activation of the fetal HPA axis leading to increased biosynthesis and secretion of cortisol. This could involve an immunological reaction to piercing fetal tissue that triggers wound repair or a reaction to the vehicle carrier, in this case vegetable oil.

It is also feasible that the increase in fetal cortisol is a result of enhanced activation of the maternal HPA axis and glucocorticoids pass from the maternal circulation to the fetus via the placenta. Measurement of cortisol in maternal blood, sampled at d90, demonstrated that there was significantly more circulating cortisol in mothers that were subjected to anaesthesia and fetal injections, twice, twenty days apart (C; $P<0.05$, TP; $P<0.01$), compared to mothers that received an intramuscular treatment (Fig. 10.5.A). Although fetal injection cohorts were assessed at d70 and not d90, the findings that maternal cortisol is increased by d90, following two injections, provide a mechanism for increased fetal cortisol that may well be measurable in the d90 fetus.

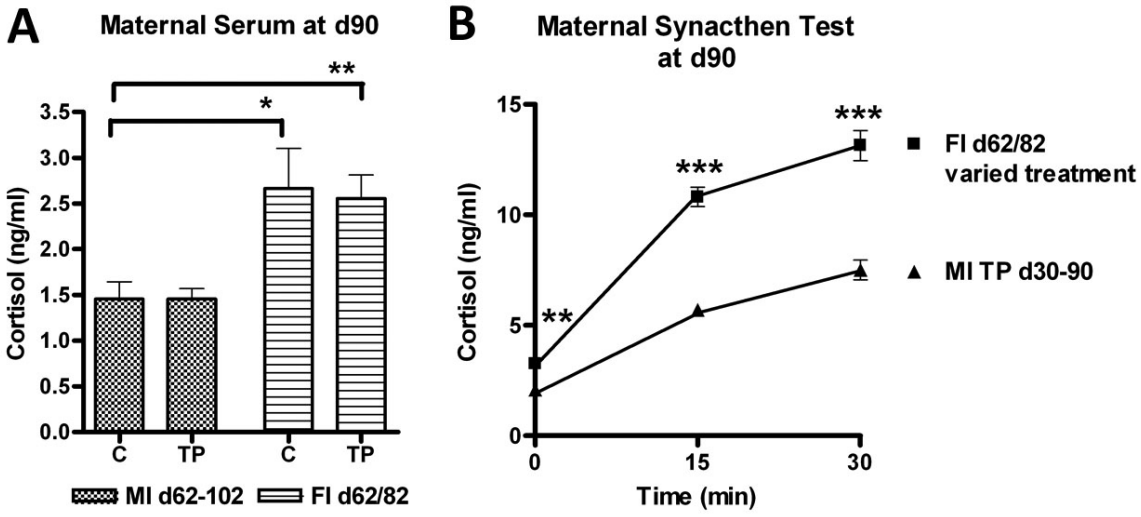

Figure 10.5 Cortisol measurements and adrenal function in pregnant ewes during the prenatal treatment period. Serum collected from pregnant mothers studied in this thesis at d90 was assayed for cortisol by RIA (A). MI: maternal injection; d62-102 C = 5, TP = 9, FI: fetal injection; d62/82 C = 4, TP = 7. A synacthen test was performed in pregnant ewes at d90 in a separate cohort of sheep, in which the treatments differed from those studied in this thesis, and cortisol measured in serum by RIA. Maternal TP treatments from d30-90 of gestation (n = 12) were compared with fetal injection treatments (n = 12) that included control, dexamethasone (synthetic glucocorticoid), diethylstilbestrol (synthetic estrogen), dehydrotestosterone (non-aromatisable androgen). Data are presented as mean ± sem and t-tests were applied between groups as indicated by bars in A) and at each time point in B). * P<0.05, ** P<0.01, *** P<0.001.

To test whether the increased maternal cortisol could be a result of exaggerated activation of the maternal HPA axis, a synacthen test was subsequently performed on a different set of experimental animals that were undergoing gestational treatments and are not included in this thesis. Pregnant ewes were assessed at d90. Maternal controls were not available to test, and fetal injections were performed at d62 and 82 and consisted of varying treatments (Fig. 10.5 legend). Nevertheless, the findings were remarkable. Similar to the observations in the cohorts described in this thesis, basal cortisol concentrations were elevated in the fetal injection group compared to the maternal treatment cohort ($P<0.01$), and considerably higher 15 min ($P<0.001$) and 30 min ($P<0.001$) after adrenal stimulation with ACTH (Fig. 10.5.B). It is not likely that maternal androgens lower cortisol output given that TP does not affect cortisol secretion compared to controls in the d62-102 treated cohort (Fig. 10.5.A). Fetal TP also had no effect on circulating fetal cortisol (Fig. 10.5.A) and it does not appear that the

varied treatments analysed in the fetal cohort in Figure 10.5.B affect cortisol secretion either since the controls animals within that group exhibited the same enhancement of cortisol secretion as observed in the other treatments.

These data are therefore good evidence that maternal cortisol concentrations are significantly raised as a consequence of the direct fetal method of treatment delivery. In addition, it is possible that the placental transfer of this steroid to the fetus is also enhanced in the fetally injected cohort. The placental expression of *HSD11B1*, which encodes 11βHSD1, was up-regulated in the fetal compared to the maternal control cohorts (Chapter 3). Since 11βHSD1 converts inactive forms of cortisol into active metabolites (424), this may allow greater amounts of cortisol to cross the placental barrier than normal. Unlike animal models of prenatal glucocorticoid exposure (286), birth weight was not affected in the fetally injected cohort, which might suggest that the endogenously raised cortisol concentrations were not in the region of exogenous cortisol concentrations administered to animals, and may have exerted more subtle effects. Nevertheless, the fetally injected offspring in this study did show possible signs of exaggerated insulin secretion in response to glucose, when compared to maternally treated control offspring. These animals also had fatty livers. Therefore there may be some indication of the presence of a metabolic phenotype that is associated with the prenatal exposure to increased cortisol, which would fit with previous research that finds adverse metabolic outcome in cases of maternal administration of glucocorticoids (277, 278, 280).

10.3.2 Implications of raised maternal cortisol and subsequent fetal phenotype

In the sheep, it is clear that injecting the fetus and/or anaesthetising the mother, during mid-gestation, results in an increase in circulating fetal and maternal cortisol. Whether or not this increase is causative, or merely associated, with the development of ovarian and metabolic anomalies in offspring is not known. These findings do however raise the point that any intervention to the mother or fetus during pregnancy, albeit small and uninvasive, may itself induce epigenetic programming that negatively impact on the fetus, without the administration of treatment. The findings generated from studies that involve fetal surgery and/or anaesthesia of the mother may therefore be confounded by the underlying effects of technical aspects of the experiment. Furthermore, these findings might have implications for

some routine procedures that are performed in pregnant women. It is not uncommon for pregnant women to receive amniocentesis mid-way through pregnancy, involving insertion of a needle through the womb and into the amniotic cavity. Chronic villus sampling is another method of fetal chromosomal diagnosis that is more invasive than amniocentesis as a biopsy of placenta is collected. Both procedures can involve the administration of a local anaesthetic, although this is not likely to have a major impact on maternal stress, rather the procedure itself could exert physiological as well as emotional stress responses (636).

10.4 Future work

The studies outlined in this thesis examine the effects of prenatal androgens immediately post-treatment in fetal life, and also in the young adult animals during the first breeding season. The changes in the adult ovary, adrenal gland and liver appear to be independent of the secondary effects of an abnormal endocrine environment and therefore provide good evidence that prenatal androgens and/or estrogens directly programme intrinsic changes in a multitude of fetal tissues. These findings have gone some way to characterise the primary origins of an adult disorder that may develop into a more clinically recognisable syndrome such as PCOS. To assess the potential contribution to the overall phenotype that might occur if the syndrome developed further there are a number of areas that would be pertinent to examine in this maternal model of androgenisation in particular.

1) Do the recognised features of PCOS arise in mid-gestation maternally androgenised offspring by the second or even third breeding season? The mid-gestation model of androgenisation is, as discussed, a more subtle model of adult reproductive and metabolic dysfunction (299, 479) and therefore may not be apparent until the animal is more mature. In addition, while PCOS can be diagnosed in the adolescent (637), the features are often confounded by environmental enhancement of the phenotype, such as obesity (638-640). In the ovine model, without such additive input, it is possible that the animals are too healthy and lean at this age to exhibit many of the features of PCOS.

2) With this in mind, it would be valuable to consider the effects of weight gain on the metabolic and reproductive features present in the 11 month maternally androgenised ewe. Studies that over-feed prenatally androgenised animals post-natally have successfully demonstrated worsening of the reproductive and metabolic phenotype (479, 482), and it would therefore be of interest to study the effects of obesity on the specific findings presented in this study.

3) In both non-obese and obese prenatally androgenised ewes, it would be useful to study whether more mature animals display hyperandrogenaemia, increased LH secretion and/or hyperinsulinaemia. At 11 months, lean animals had the potential or predisposition to have raised basal peripheral concentrations of all three hormones, however this was

not yet realised. The main questions would be whether alterations to the hormonal environment are additive or alter some of the phenotypes described in this study, such as thecal cell, liver and adrenal function. It is realistic to hypothesise that it is the combination of all of these environmental factors with the intrinsic features studied in this project that will lead to the development of an animal that fulfils the diagnostic and anatomical features that are present in PCOS women.

It is clear from the studies presented in this thesis, that the programming effects of prenatal androgens on the adverse outcomes in the adult, might not be an effect of androgens *per se*, and that there are multiple ways that this programming could occur. The effects of various physiological stressors during pregnancy are well documented to have deleterious effects in infants and adult progeny in both humans and animals (262, 274). These include over and undernutrition, dietary composition changes, emotional stress and the fetal/maternal exposure to toxins and steroid hormones (265, 278, 280, 284, 286, 518, 520, 641-646). It is demonstrated in this thesis that the fetal injection method results in increased exposure of the mother, and potentially the fetus, to elevated levels of cortisol, and this is associated with an adult phenotype including reproductive and metabolic anomalies similar to the maternal model of prenatal androgenisation. It is therefore important to dissect out the underlying mechanisms that have led to this phenotype in the adult sheep and whether cortisol exposure can be directly linked to this.

1) One of the major unknowns emerging from this study is whether it is the physical effect of injecting the fetus, or the impact of maternal anaesthesia that could influence maternal and/or fetal homeostasis to trigger a stress response. Indeed, particularly high levels of cortisol are observed in the mothers that received this treatment. One way to investigate this would be to sedate the pregnant ewe twice during gestation at d62 and again at d82, without performing any fetal injections and assess the effect of this relatively unobtrusive intervention on maternal cortisol secretion, the immediate effect on fetal developmental parameters and the long-term effect in the adult offspring of these mothers.

2) In order to dissect whether it is a fetal or maternal source of cortisol that confers these effects, a set of subtraction experiments could be performed. To determine if it is maternal cortisol that is associated with this phenotype, endogenous cortisol could be

initially controlled by immunising the animal against ACTH and then constantly replacing cortisol exogenously, so that the physiological levels are maintained. The pregnant ewe would then be anaesthetised at d62 and d82 and receive a control fetal injection simultaneously. This would prevent endogenous activation of the maternal HPA axis as a consequence of the physical interventions. If the adult phenotype is reversed through inhibition of the maternal HPA axis in this manner, then this would confirm that the phenotype is associated with secondary effects in the mother that may be mediated at the level of the placenta (647). If the phenotype is present in adult offspring then this is likely mediated through the activation of the fetal HPA axis.

Alternatively, this could be studied directly in the fetus, by anaesthetising the mother and directly injecting the fetus with an inhibitor of cortisol synthesis such as metyrapone. This would identify if the phenotype arises from the exposure of the fetus to cortisol (that could come from the fetus or mother), if the phenotype was subsequently not observed in the fetal injection group plus cortisol inhibitor. By inducing raised endogenous cortisol through these treatment interventions and not exposing the mother to exogenous glucocorticoids such as dexamethasone, this would generate a model that examines the effects of physiological levels of this stress hormone on fetal development that could realistically occur in everyday life.

10.5 Conclusion

The findings presented and discussed in this thesis highlight the remarkable plasticity and therefore vulnerability of the fetus to changes in the maternal and/or fetal environment. The Barker hypothesis highlights just how susceptible the developing conceptus is to *in utero* programming effects which can lead to adult pathophysiologies (261, 262), including reproductive and metabolic anomalies that are associated with PCOS. The main findings from this study suggest that there are multiple effects of *in utero* programming on the developing fetus that pre-dispose offspring to develop the adult characteristics of PCOS. These features are likely to manifest in a clinical sense, either passively as the animal matures, or in circumstances in which the external environment influences such traits. This is of particular significance given the widespread incidence of poor lifestyle and obesity that is common today. Furthermore, it is evident that the underlying causes of disorders such as PCOS are not exclusively an effect of increased *in utero* exposure to sex steroids but could include a range of adverse fetal environments, including inappropriate exposure to the stress hormone cortisol.

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Appendices

Appendix A: RIA protocols

Progesterone Assay	Sample or STD (µl)	PGBS Buffer 0.1M pH7.4 (µl)	Antibody (µl) 1:6000	Tracer (µl) ~12,000 cpm
To				100
NSB		290 (+10 stripped plasma [SP])		100
Bo		190 (+10 SP)	100	100
Standards 4-1000pg/100µl	**50**	140 (+10 SP)	100	100
Sample/Unknown	**10**	190	100	100

Cortisol Assay	Sample or STD (µl)	Citrate Buffer 0.1M pH4.0 (µl)	Antibody (µl) 1:100,000	Tracer (µl) ~12,000 cpm
To				100
NSB		200 + 90 (+10 SP)		100
Bo		100 + 90 (+10 SP)	100	100
Standards 1-2000pg/100µl	**100**	90 (+10 SP)	100	100
Sample/Unknown	**10**	190	100	100

Testosterone Assay	Sample or STD (µl)	PGBS Buffer 0.1M pH7.4 (µl)	Antibody (µl) 1:600,000	Tracer (µl) ~12,000 cpm
To				100
NSB		300		100
Bo		200	100	100
Standards 0.25-500pg/100µl	**100**	100	100	100
Sample/Unknown	**100**	100	100	100

Androstenedione Assay	Sample or STD (µl)	PGBS Buffer 0.1M pH7.4 (µl)	Antibody (µl) 1:320,000	Tracer (µl) ~12,000 cpm
To				100
NSB		300		100
Bo		200	100	100
Standards 0.25-500pg/100µl	**100**	100	100	100
Sample/Unknown	**100**	100	100	100

Ovine LH Assay	Sample or STD (µl)	Phosphate Buffer 0.05M (µl)	Antibody (µl) 1:120,000	Tracer (µl) ~20,000 cpm
To				100
NSB		300		100
Bo		200	100	100
Standards 10-5000pg/100µl	**100**	100	100	100
QCs	**100**	100	100	100
Sample/Unknown	**100**	100	100	100

Ovine FSH Assay	Sample or STD (µl)	Phosphate Buffer 0.05M (µl)	Antibody (µl) 1:12,000	Tracer (µl) ~20,000 cpm
To				100
NSB		300		100
Bo		200	100	100
Standards 5-2500pg/100µl	**100**	100	100	100
Sample/Unknown	**100**	100	100	100

Ovine E2 Assay	Sample or STD (µl)	PGBS Buffer 0.1M pH7.4 (µl)	Antibody (µl) 1:7.5	Tracer (µl) ~12,000 cpm
To				100
NSB		300		100
Bo		200	100	100
Standards 0.06-64pg/100µl	**100**	100	100	100
Sample/Unknown	**200**	200	100	100

Multispecies Leptin Assay	Sample or STD (µl)	Buffer Supplied (µl)	Antibody (µl) ready to use	Tracer (µl) ~14,000 cpm
To				100
NSB		300		100
Bo		200	100	100
Standards 100-5000pg/100µl	**100**	100	100	100
QCs	**100**	200	100	100
Sample/Unknown	**200**	200	100	100

Appendix B: 'Data not shown' from Chapter 4

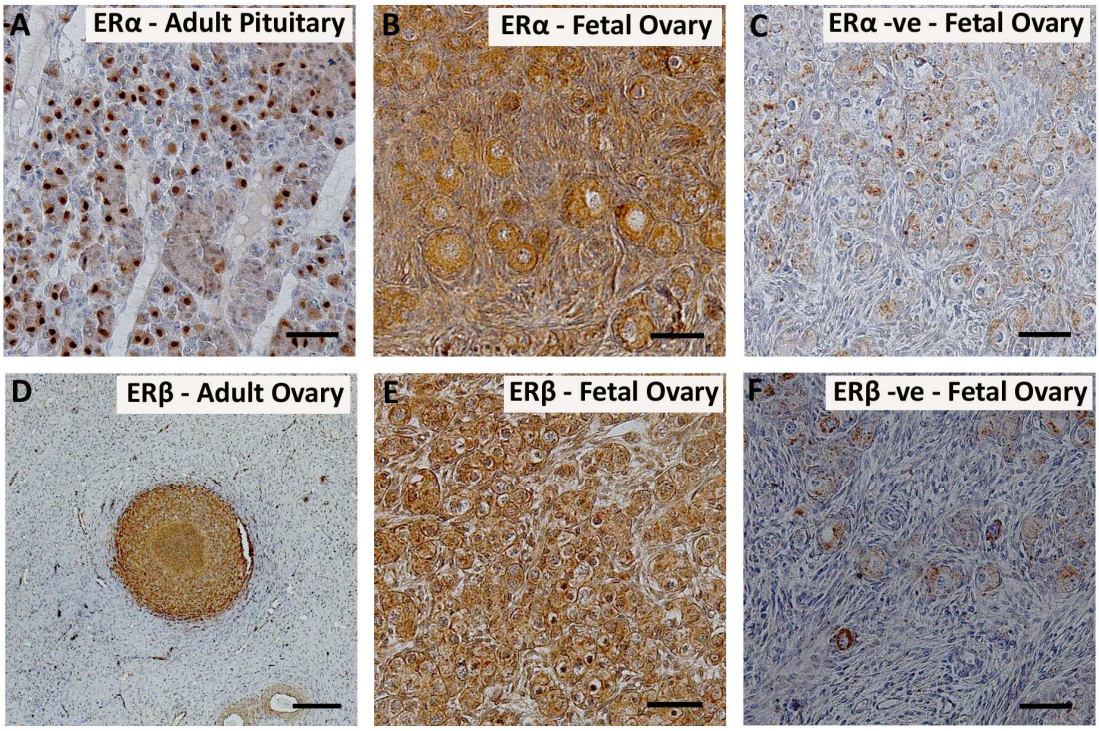

Figure AB.1 Immunohistochemistry for ER antibodies in sheep tissues. ERα antibody was strongly localised to the nuclei in cells of the adult sheep pituitary (A; 1:100, no tyramide enhancement required). However, ERα appeared to be non-specifically expressed throughout the ovarian stroma of the fetal ovary and this staining was not nuclear (B; 1:30, tyramide). Control sections for ERα immunolocalisation were not completely negative (C, tyramide). Similarly, ERβ staining was demonstrated in the nucleus of granulosa cells of a small follicle in an adult sheep ovary (D; 1:50, no tyramide), and was strongly (E; 1/30, tyramide) but not specifically (F, tyramide) immunolocalised in fetal ovaries. Scale bars are all 50µm, except D) which is 200µm.

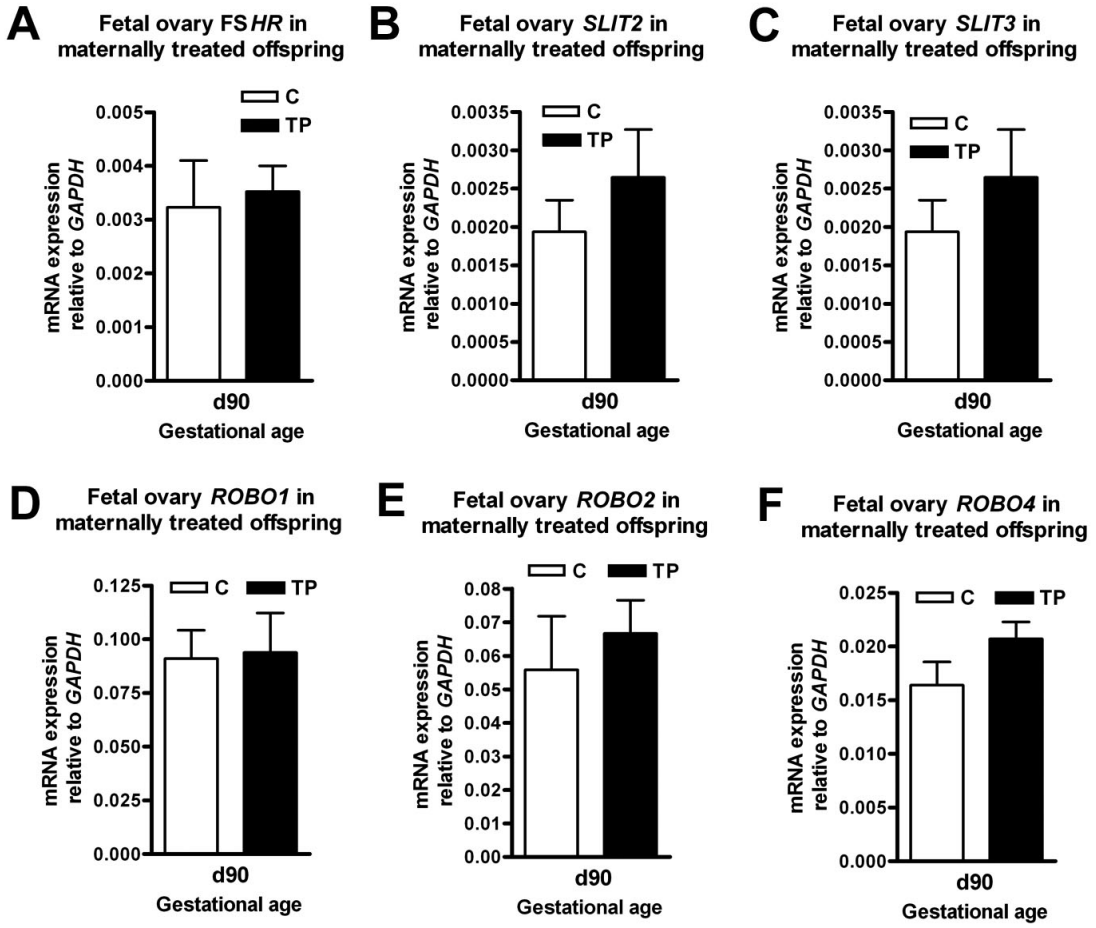

Figure AB.2 Gene expression in the d90 ovary of maternally androgenised fetuses. There was no change in the ovarian expression of FSHR (A), SLIT2 (B), SLIT3 (C), ROBO1 (D), ROBO2 (E), or ROBO4 (F).

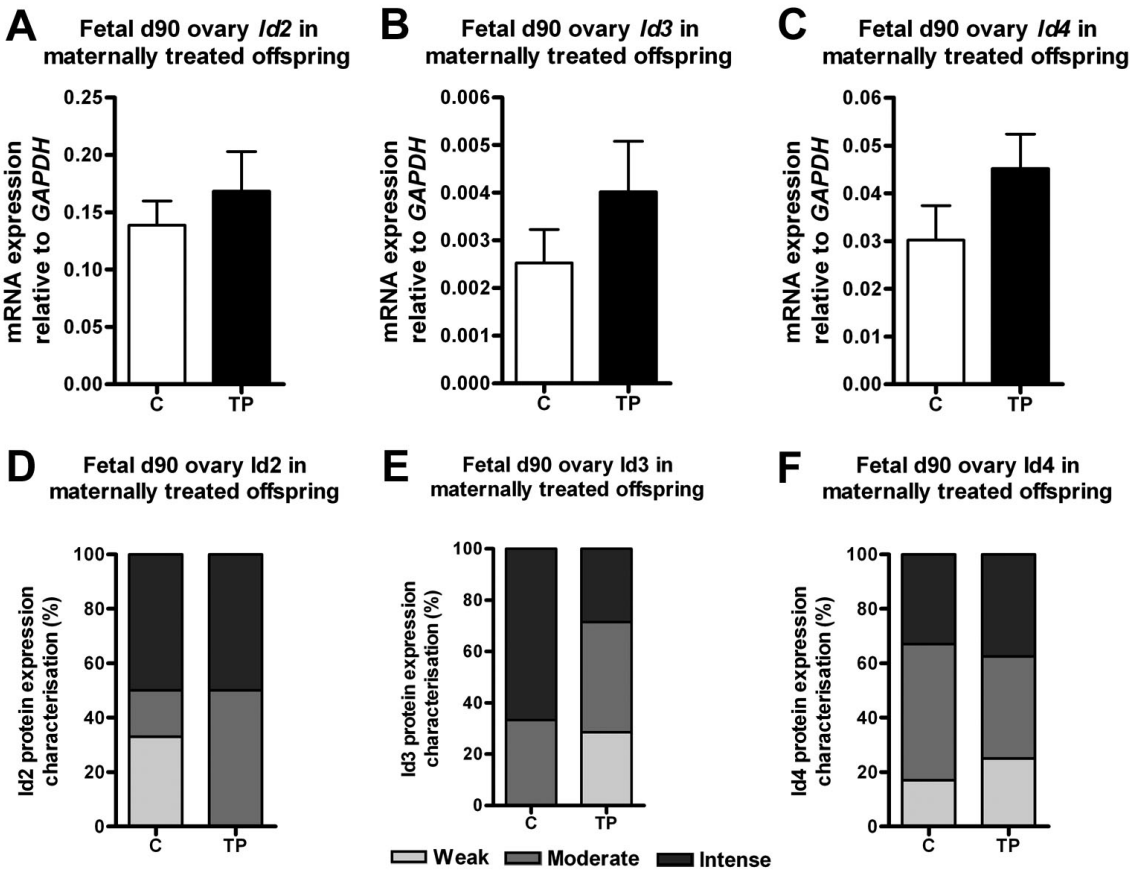

Figure AB.3 Id gene and protein expression in the d90 ovary of maternally androgenised fetuses. There was no change in Id2 (A), Id3 (B) or Id4 (C) mRNA expression, or Id2 (D), Id3 (E) or Id4 (F) protein expression in these ovaries.

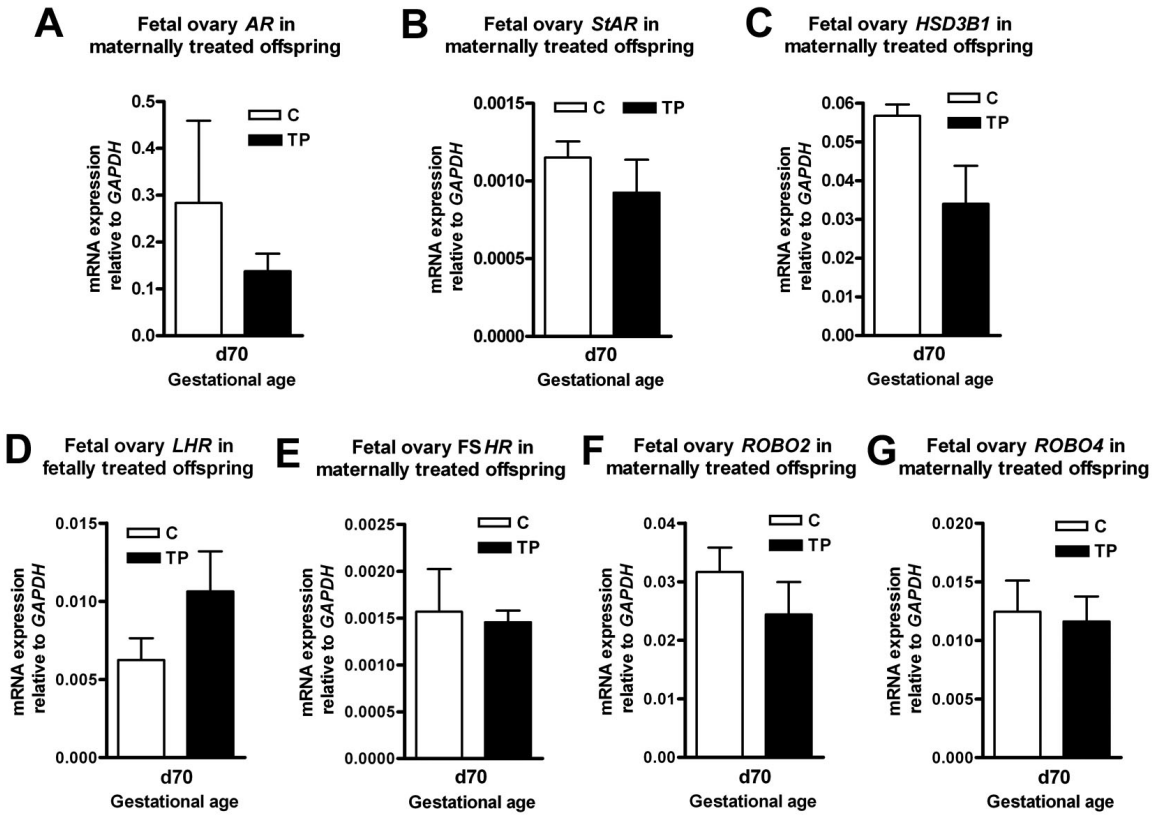

Figure AB.4 Gene expression in the d70 ovary of maternally androgenised fetuses. There was no change in the ovarian expression of *AR* (A), *StAR* (B), *HSD3B1* (C), *LHR* (D), *FSHR* (E) *ROBO2* (F) or ROBO4 (G).

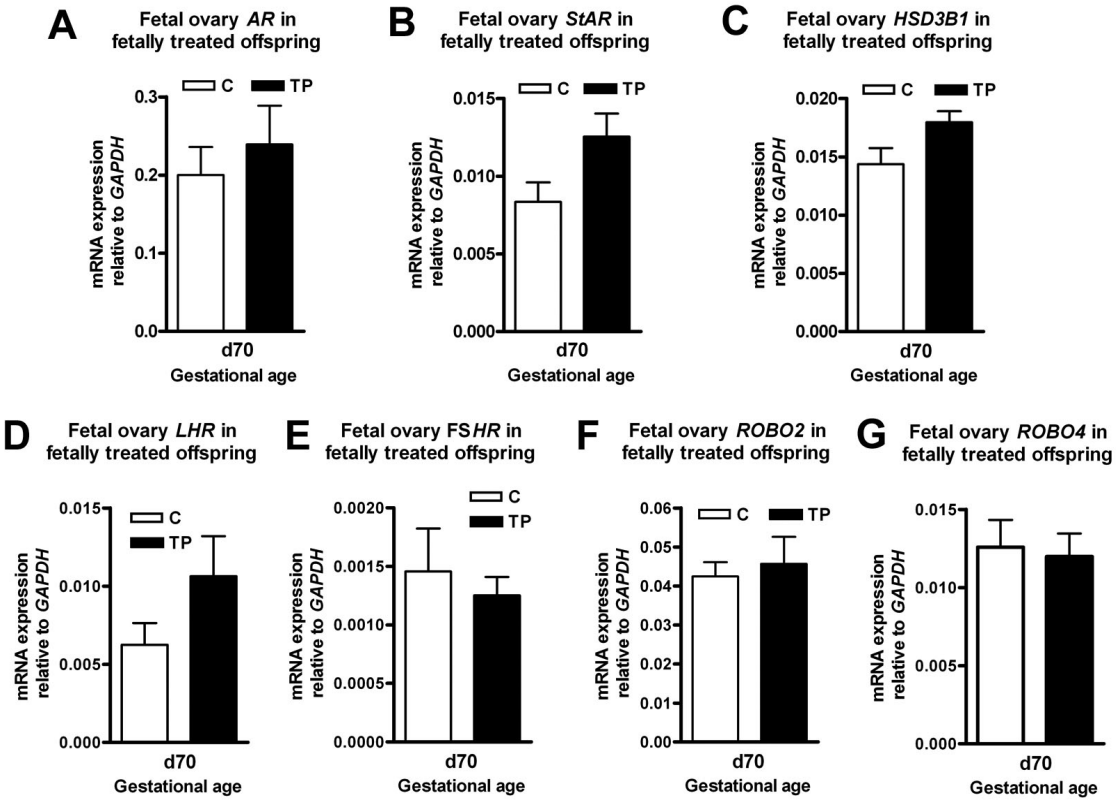

Figure AB. 5 Gene expression in the d70 ovary of fetally androgenised fetuses. There was no change in the ovarian expression of *AR* (A), *StAR* (B), *HSD3B1* (C), *LHR* (D), *FSHR* (E) *ROBO2* (F) or ROBO4 (G).

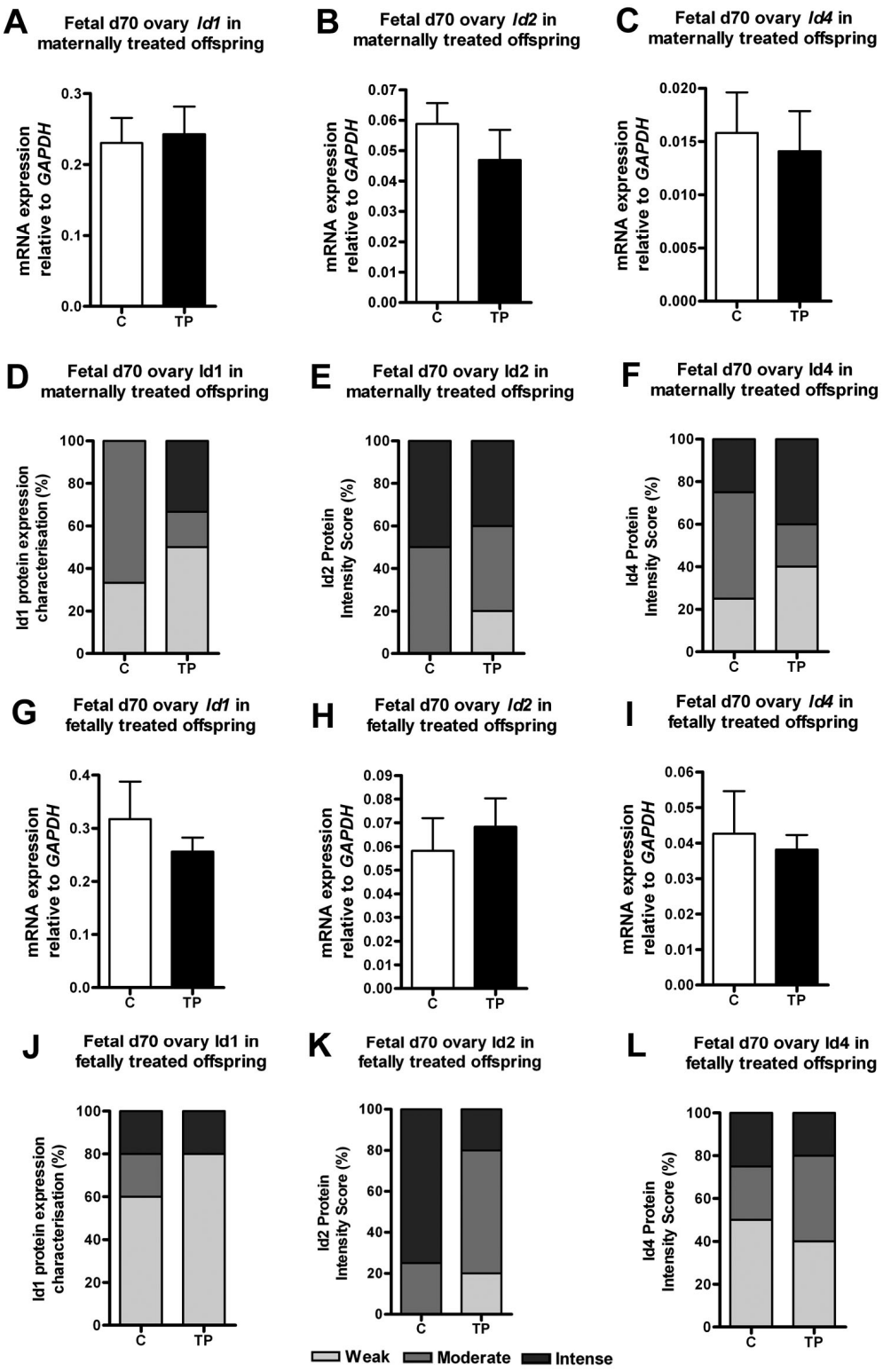

Figure AB.6 Id gene and protein expression in the d70 ovary of androgenised fetuses. In maternally and fetally treated fetuses, there was no change in Id1 (A and G), Id2 (B and H) or Id4 (C and I) mRNA expression, or Id1 (D and J), Id2 (E and K) or Id4 (F and L) protein expression in these ovaries.

Appendix C: 'Data not shown' from Chapter 9

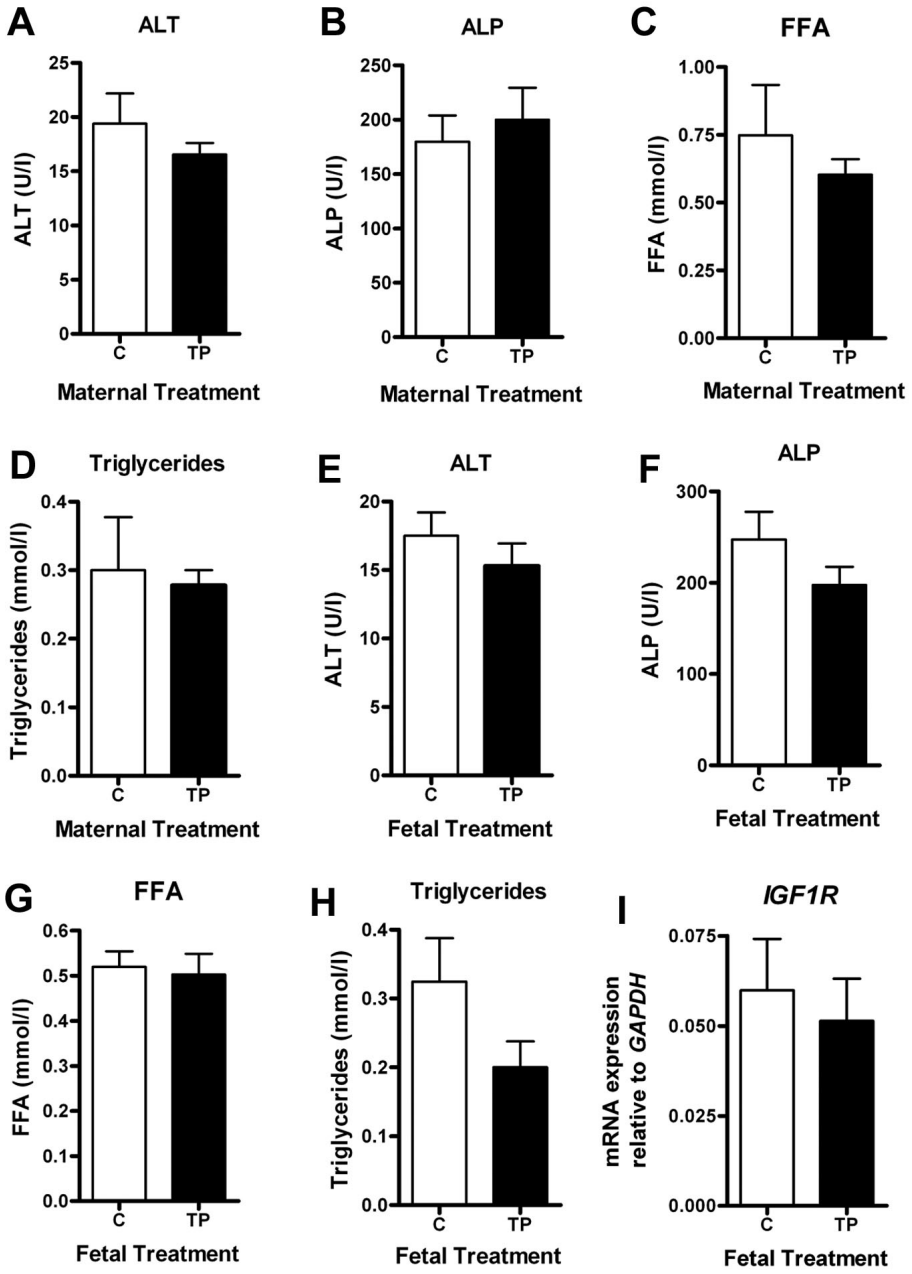

Figure AC. 1 Data not shown from Chapter 9. Peripheral plasma analytes are shown for maternal (A-D) and fetal (E-H) androgenised cohorts for ALT, ALP, FFA and triglycerides, respectively. I) mRNA expression for *IGF1R* in livers from the fetally injected cohort.

Appendix D: Publications relating to this thesis

Hogg K, Etherington SL, Young JM, McNeilly AS, Duncan WC (2010) **Inhibitor of Differentiation (Id) Genes Are Expressed in the Steroidogenic Cells of the Ovine Ovary and Are Differentially Regulated by Members of the Transforming Growth Factor-β Family.** Endocrinology 151:1247-1256

Hogg K, McNeilly AS, Duncan WC (2011) **Prenatal Androgen Exposure Leads to Alterations in Gene and Protein Expression in the Ovine Fetal Ovary.** Endocrinology 152:2048-2059

Hogg K, Wood C, McNeilly AS, Duncan WC (2011) **The *in utero* programming effect of increased maternal androgens and a direct fetal intervention on liver and metabolic function in adult sheep syndrome.** PLoS One doi.10.1371/journal.pone.0024877